THE ROLE OF TRANSDUCTION AND PSEUDOLYSOGENY IN BACTERIO-PHAGE-HOST INTERACTIONS IN A NATURAL FRESHWATER ENVIRONMENT

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CHAPTER I

INTRODUCTION

Bacteriophages have long been disregarded as potential mediators of microbial activities in natural waters due to their insignificant population densities. However, in 1989, Oivind Bergh and co-workers at the University of Bergen in Norway reported that bacteriophage numbers in ocean waters were three to seven times higher than what was previously assumed (6). Observations of samples using transmission electron microscopy revealed as many as 1×10^8 bacterial viruses per milliliter of water. The implications of this simple observation forever ended the conventional wisdom that phage infection of bacteria in natural waters was an extremely rare event. And, in fact, our laboratory has since shown that transduction, the virally mediated transfer of genetic material, is prevalent in freshwater microbial communities and that both plasmid and chromosomal DNA can undergo transfer (30, 49, 50, 67, 71, 76). It has now been hypothesized that transduction may be the predominant form of bacterial gene transfer in natural environments.

With our heightened knowledge of bacteriophage-host cell interactions in natural environments comes new concerns of the potential for risk associated with the application of genetically-engineered microorganisms (GEMs) to

environmental ecosystems. GEMs specific for such tasks as biodegradation, wastewater treatment, and mineral processing are now being utilized in many natural environments. The frequency of such applications will only increase in the future. However, apprehension arises over the fact that many GEMs are derived from indigenous environmentally competent bacteria, and, consequently, whatever interactions occurring among these native populations may potentially embody GEMs as well. Do we possess sufficient knowledge and data to assure, either now or in the distant future, that gene transmission between GEMs and indigenous microbial communities will not occur, and if it perhaps does, that it will not have deleterious effects? Caution is warranted, and many more experiments need to be conducted before concerns can be adequately addressed.

It was the purpose of this study to better understand phage-host interactions at a level more closely associated with the natural environment. Previous studies, by necessity, relied on well-fed, laboratory-grown host strains. It is well known that in environmental ecosystems, bacterial populations subsist under severely starved conditions. By using continuous-culture chemostats, we were able to more closely mimic natural starvation states. Microcosms were also directly established in a freshwater lake as a means of duplicating the environmental parameters that would actually impinge on a natural community. *Pseudomonas aeruginosa*, common to freshwater environments, and its phage, UT1, directly isolated from a freshwater lake, were chosen as a model host-parasite system for this study.

As a result of these studies, we have encountered a unique phage-host interaction referred to as pseudolysogeny. This phenomenon was first observed in 1915 by Twort (85), although its implications were not recognized by him at the time, and ten years later actually distinguished from true lysogeny by McKinley (42). It has since been relatively ignored. Pseudolysogeny refers to a bacteriophage-host cell interaction where the nucleic acid of the phage, upon infection of an appropriate host cell, does not integrate itself into the host's genome or establish itself as an autonomous replicon, as would occur in a typical lysogenic response. Nor does the phage initiate self-replication and cell lysis, as it would in a lytic response. Rather, the phage nucleic acid simply resides within the cell in a non-active form. It is hypothesized that, due to the cell's highly starved state, there is insufficient energy available for the phage to initiate a true lysogenic or lytic response. However, upon nutrient accruement, the phage acquires the necessary energy and undergoes either true lysogeny or replicates and lyses its host.

An interaction such as pseudolysogeny adds another potential risk associated with the environmental application of GEMs because pseudolysogeny will lengthen the half-lives of phage genomes in the environment. That is to say that bacteriophages, whether virulent or temperate, can initiate pseudolysogeny and sustain their genomes in a relatively protected state for extended periods. These phages also remain undetected, since the cells they inhabit do not appear lytic or lysogenic. Consequently, phage reservoirs are maintained at high levels, and, when nutrients become avail-

able, the potential occurrence of horizontal gene transfer mediated by transduction increases dramatically.

CHAPTER II

LITERATURE REVIEW

Bacteriophage genetics. Phage infection of a bacterial cell is a random event, with potential host organisms being identified by simple diffusion of the phage through the medium until a collision occurs (15). If the cell has an appropriate receptor, the phage will irreversibly bind and inject its nucleic acid (15). Traditionally, it has been assumed that one of two possible productive responses then transpires - the lytic or the lysogenic response (8, 15, 40). The lytic response, elicited by a virulent or temperate phage, results in the synthesis of progeny phage particles utilizing the host cell's own synthetic machinery, followed by cell lysis. The number of progeny phage virions released constitutes the burst size, which can conceivably approach thousands of phages per cell. In the lysogenic response, the phage genome becomes integrated in the host chromosome or exists within the cell in a plasmid-like form. Production of a repressor protein suppresses expression of most or all of the phage DNA, thus inhibiting a typical lytic cycle. Presence of the repressor protein also renders the cell immune to infection by the same or a closely related phage. Under certain stresses, however, the latent phage DNA, referred to as the prophage, reactivates and the lytic response is initiated.

Bacteriophages capable of instituting a lysogenic response are designated as temperate phages.

Transduction. Cellular infection by bacteriophages leads to the gene-exchange mechanism known as transduction (90). Transduction is generally mediated by temperate phages and occurs in two distinct forms, generalized or specialized (30, 50). In generalized transduction, host chromosomal or plasmid DNA is mistakenly packaged into progeny phage particles. Upon subsequent cell lysis, these unique transducing particles go on to infect other hosts, where their transduced genome either recombines with the host chromosome, or in the case of plasmid DNA, establishes itself as an extrachromosomal replicon in the new host cell. In both cases, a transductant with an altered phenotype is produced.

Specialized transduction functions similarly to generalized transduction except that only specific portions of the host DNA are taken up through imprecise excision of the prophage. By necessity, these segments must lie on either side of the prophage integration site. Progeny phages then contain a combination of viral and bacterial genetic material. Some of these phages are able to successfully infect new hosts while others remain defective.

Recent studies have demonstrated that transduction is a viable gene-exchange mechanism exhibited by indigenous microbial populations in freshwater and marine ecosystems (28, 44, 49, 54, 62). Significant transductional interactions rely on sufficient concentrations of both bacteria and phage (15). Bacterial numbers in freshwater environments routinely range

from 10^4-to-10^7 cells/ml (10, 75). Phage counts are estimated to approach 10^8-to-10^{10} particles/ml (6). Thus the apparent potential for interaction is considerable.

That transduction is a probable occurrence in freshwater habitats was confirmed by experiments performed by Saye *et al.* (75) utilizing the generalized transducing phage F116 of *P. aeruginosa* (48). F116 lysogenic donor cells carrying the conjugation-deficient plasmid Rms149 (19) were mixed with appropriate recipient cells in an *in situ*-incubated environmental test chamber. Transductants were recovered. It has also been shown that chromosomal DNA is likewise adept at undergoing transduction under similar conditions (71, 76). Additionally, Replicon *et al.* (67) has shown that transduction can maintain a less-fit phenotype within a genetically heterogenous bacterial population.

Since bacteria in nature commonly subsist under starvation conditions (52, 61, 73), current research has taken into account the effects of limited nutrient supply on transductional processes. Attachment of phage virions to starved cells has been found to not be impaired, but upon infection both an increased latency period and a diminished burst size are observed (32). These experiments also indicate that in nutrient-depleted hosts, the virulence of lytic phages is significantly reduced. Average phage half-lives under these conditions only extends from 12-to-24 hours (32).

Pseudolysogeny. Pseudolysogeny may be the mechanism utilized by phages to sustain their populations in natural environments (32, 59, 72). In such a case, the phage genome endures for extended periods of starvation by

coexisting with the bacterial host, which, through evolutionary modifications, has adapted itself for survival under harsh environmental conditions (73). As nutrient supplies become more favorable, the pseudolysogens induce true lysogeny or activate the lytic response, resulting in the release of progeny virions. Lysogens of the temperate phage F116 combined with a starved *P. aeruginosa* strain in simulated freshwater microcosms showed low levels of phage production until the addition of 10^{-5}% yeast extract, whereupon lysogenization increased dramatically (Gaitonde and Miller, unpublished observations). Starved *Escherichia coli* cells show a similar response (35). When *P. aeruginosa* cells were directly isolated from a lake, fully 70% were found to contain phage-specific homologous DNA sequences when probed with nucleic acid from environmentally prevalent phages (58). However, of these, only 1-7% actually exhibited lysogenic characteristics when scored using a sensitive bacterial indicator strain.

Pseudolysogeny may also account for the high numbers of virulent phages found in nature. Under cell starvation conditions, it seems unlikely that a virulent phage, incapable of latent existence within its host, could sustain itself in appreciably large numbers. Experiments were conducted in which UT1, a virulent freshwater *P. aeruginosa* phage (59), was mixed with indigenous bacterial hosts in lakewater microcosms sustained over a 45 day period (59). Bacterial densities were found to initially decrease but then stabilize when UT1 was present. Colony hybridizations of host cells using phage UT1 DNA as a probe indicated that 45% of the total recoverable colonies contained UT1 genetic material. This suggested that in nature an equilibrium between

host and phage can be implemented, such that the virulent phage does not eradicate its host, but rather coexists with it until more favorable conditions are attained.

Environmental ultraviolet irradiation. The ultraviolet (UV) radiation spectrum is divided into three sections based on wavelength: UV-A (320-400 nm), UV-B (290-320 nm), and UV-C (100-290 nm). Radiation reaching the earth's surface consists predominantly of UV-A and some UV-B, while the ozone layer absorbs all UV-C (88).

DNA is the primary site of damage inflicted by UV irradiation (12). UV-C causes the formation of pyrimidine dimers, as does UV-B (but in significantly lower yields). Exposure of DNA to UV-B mainly leads to single-strand breaks. In ocean waters, UV-B has been shown to effectively induce DNA damage within the upper 2-6 m, depending on water clarity (65, 79).

Repair of DNA damage occurs through the SOS system, which has been studied predominantly in *E. coli* (60). Upon DNA damage, a chromosomally encoded gene designated *recA* is activated. Its gene product promotes recombinational repair of the damaged DNA and also, by cleaving LexA, the negative regulator of the SOS pathway, induces this regulon. Activation of the SOS network leads to the expression of approximately twenty genes.

Many prophages are activated to lytic growth following exposure of the host cell to DNA-damaging agents. In the environment, the predominant damaging agent is UV radiation (5). The prophage of the *P. aeruginosa* phage F116 has been shown to be UV inducible (76). Several

recombination-deficient, *recA* mutants of *P. aeruginosa* have also been shown to exhibit reduced efficiencies of lysogenization with phage F116. In these mutants, lysogeny is established at much higher multiplicities of infection (MOI) than in their wild-type parents (45).

Bacteria in the environment. The most important environmental factor affecting a microorganism is the availability of energy, and, since most ecosystems are oligotrophic, energy resources are rarely abundant (53). Carbon concentrations in natural environments typically approach only 1 to 15 mg/l (86). As a result, most of the bacteria in any given ecosystem are subsisting in a starved state, and are essentially inactive.

A number of laboratories have studied starvation effects in marine *Vibrio*, and have elucidated common stages of starvation survival in this species (53). In stage 1 (0-14 days) large fluctuations in viable cell counts are observed along with fluctuations in protein, DNA, and RNA concentrations. Oxygen uptake measurements indicate intense metabolic activity, as would be expected since all endogenous energy reserves are being used. During stage 2 (14-70 days), viable cell numbers decrease and the DNA per cell drops to between 4 and 8% of the original amount. Protein concentrations drop similarly while RNA concentrations exhibit only slight decreases. Cells lost over 90% of their original volume converting from rod to coccoid morphology. It has been suggested that this is a sporelike, "somnicell" or "ultramicrocell" stage for non-sporulating bacteria (73). Ultramicrocells have been observed in several bacterial species both in aquatic and soil environments (53) and are consid-

ered prime indicators of a population undergoing starvation. In stage 3 (70-98 days) viable cell counts stabilize at low levels. However, acridine-orange direct counts (AODC), where actively growing cells fluoresce red/orange and inactive cells fluoresce green, remained fairly high. Inactive cells in such a state are referred to as viable but nonculturable (73). These cells, under low nutrient conditions, are maintained in a metabolically active state but will not form colonies when plated. It has been suggested that the viable but nonculturable condition is the conventional state of bacterial populations in environmental ecosystems (73). Generation times of such inactive cells in aquatic environments have been shown to be upwards of 200 hours (24). Total microbial concentrations, utilizing AODC, have been estimated at 10^6-to-10^7 cells/ml of fresh water and 10^4-to-10^6 cells/ml of ocean water, of which 80% may, on average, be considered inactive (50, 73).

Bacteriophages in the environment. The concentration of bacteriophages in environmental ecosystems was considered negligible until recently. However, due to improved counting techniques, it is now known that phages are significant constituents of virtually all ecosystems. Several studies report phage abundances from 10^3-to-10^8 particles/ml in lakes and ocean waters (50). Replicon and Miller (68) have observed that a milliliter of lakewater can contain as many as 10^4 plaque forming units (PFU) of exclusively *P. aeruginosa*-specific phages. Phage-to-bacterium ratios (PBRs) have been observed to be as high as 50:1 in freshwater environments, implying that most of the cells are infected by phages. Bergh *et al.* (6) estimated that one

third of the bacterial populations in various freshwater and marine environments may undergo phage attack each day. Proctor and Fuhrman (64), using electron microscopy to observe phage infected cells, predicted that up to 70% of the cells in marine environments may be infected at any one time. More recently, Suttle (82) estimated that up to 50% of these marine microbial populations must be lysed each day in order to maintain observed bacteriophage concentrations.

A large number of environmental factors affect phage viability and infectability. Most studies have concentrated on enteric viruses which have been shown to significantly decay over a period of only a few days in marine and freshwater environments (17). Phage deactivation in such instances was found to be temperature dependent and was enhanced by sunlight and sewage pollution (7). Estimates of decay rates of natural marine bacteriophages under numerous conditions were made by Suttle and Chen (83). Flagellates were shown to consume viruses at the rate of 0.15/hour. Solar radiation produced considerable decay rates of 0.4 to 0.8/hour, while in the absence of UV-B, rates decreased to 0.17/hour. Estimates of decay rates of freshwater phages were made by Saye *et al.* (75) and Ogunseitan *et al.* (59), who both showed that phage half-lives decreased in the presence of the indigenous freshwater community. The *P. aeruginosa* phage UT1, for example, maintained an average half-life of 29 hours in autoclaved lake water but decreased considerably to 18 hours when incubated in natural lake water.

Temperature also plays a major role since it influences phage and cell mobility (15). More importantly, temperature has been shown to alter phage receptors on host cells which then become insensitive to phage attack (11).

Continuous culture. Cells grown in a batch culture steadily attain a maximum growth rate followed soon after by a steady rate of cell death due to the accumulation of waste products, exhaustion of nutrients, changes in pH, and numerous other less significant factors. A chemostat circumvents this typical death phase by maintaining populations at a constant size by continuous dilution (41). This is accomplished by the addition of fresh medium into the culture at a defined, constant rate with the simultaneous removal of an equal volume of the culture. The ratio of the rate at which fresh medium is added (F; liters/hour) to the volume of the culture (V; liters) is termed the dilution rate (D; $hour^{-1}$), and is defined by:

$$D = \frac{F}{V}$$

The dilution rate represents the number of volumes of medium that pass through the culture vessel in one hour. The reciprocal of the dilution rate 1/D, denotes the generation time (τ):

$$\tau = \frac{1}{D} = \frac{V}{F}$$

The generation time refers to the average time in hours it takes for the culture vessel to be completely replenished with medium.

As cells grow in the chemostat they are continuously being washed out of the culture vessel. The net change in the number of cells over time is determined by the rate of growth and washout:

$$\frac{dN}{dt} = \mu N - \frac{F}{V}N$$

where μ represents the specific growth rate of the culture per hour and N the concentration of cells per milliliter. For population size to remain constant, it is necessary that the net change in the number of cells with time be zero:

$$\frac{dN}{dt} = 0$$

and thus,

$$\mu N = \frac{F}{V}N$$

and,

$$\mu = \frac{F}{V}$$

Therefore, by maintaining a constant volume in the culture vessel and changing the rate at which nutrient is added, it is possible to control the specific growth rate of the population.

Two variables which can significantly affect chemostat growth functions are sampling and wall growth. Large samples removed from the culture vessel may reduce total volume, thus affecting the dilution rate and ultimately the metabolism of the cells. Wall growth commonly occurs when dense cell

cultures are maintained over extended periods. In such a case, cells adhering to the glass vessel may represent a large portion of the total cellular population, thus affecting steady state dynamics. Neither variable was a factor in the experiments reported in this study, however, since sampling size was small and cultures were maintained at low population densities.

CHAPTER III

TRANSDUCTION OF A FRESHWATER MICROBIAL COMMUNITY BY BACTERIOPHAGE UT1

Introduction

Transduction, the transfer of genetic material via bacteriophages, is a well known phenomenon routinely used in the laboratory for procedures such as gene mapping and strain construction (28). Only recently has the importance of this method of gene transfer to microbial genetic diversity and evolution in natural habitats become apparent (50). In the past, transduction had often been discounted as a potentially important process for the redistribution of genetic information (both chromosomal and extra-chromosomal) in bacterial populations because it is reductive (i.e., the donor is killed in the process of donating its genetic material to the recipient). However, recent reports have documented that transduction can be a fertile gene exchange system in natural ecosystems (28, 30, 43). Transduction has now been shown to be a significant mediator of gene transfer within several natural ecosystems, including soils (80, 81, 89), plant surfaces (27), freshwater environments (43, 54, 75), marine environments (62), and animals (4, 25, 55, 56).

Our laboratory has been using *P. aeruginosa* PAO as a model system to study virus-mediated gene transfer in freshwater microbial populations (43, 44,

49, 54, 75, 76). Our studies have revealed a significant potential for transduction of both plasmid and chromosomal DNA in these environments mediated by the well-characterized *P. aeruginosa* transducing phage F116 (47, 48) and the closely related phage øDS1 (75, 76). However, neither of these phages were isolated from aquatic environments and have existed as laboratory strains for several years.

In an attempt to determine if naturally occurring transducing phages are capable of mediating gene transfer in *P. aeruginosa*, we isolated several phages from a freshwater lake in eastern Tennessee and have studied one, UT1, in some detail (58, 59). This phage infects both natural isolates of *P. aeruginosa* and well-characterized laboratory strains such as PAO. Plaques formed by this phage upon initial isolation were turbid, but only clear plaques are observed when the phages were plated on laboratory-grown hosts. Southern analysis of the genome of this phage revealed that it shares significant homology with the virulent bacteriophage E79 (58, 59). These data suggest that although UT1 is apparently virulent on well-fed hosts, starvation conditions found in the aquatic habitat favor the establishment of a pseudolysogenic relationship between the bacteriophage and its host bacterium, much like the one described by Romig and Brodetsky (72) in soil bacilli. In some aquatic environments as many as 45% of the presumptive *P. aeruginosa* isolates show evidence of pseudolysogeny for phage UT1 by colony hybridization with a UT1-specific DNA probe (43, 59).

The purpose of the present study was to determine if phage UT1 was capable of mediating transduction. Because mutants of E79 with reduced

virulence for the host have been shown to be capable of mediating transduction (51), we felt that this was a distinct possibility. We also wished to utilize a natural lakewater microbial community as recipients in UT1-mediated transductions to demonstrate the potential for transduction to occur in a system in which both transducing phages and recipient bacteria are members of a natural lakewater community.

Materials and Methods

Bacterial strains, bacteriophages, and plasmids. All laboratory strains used in this study were derived from *P. aeruginosa* PAO (Table 1). Bacteriophage F116 is a temperate, generalized transducing phage (36, 47, 48). Phage UTI was isolated from natural lakewater and identified as belonging to the family *Myoviridae* (58, 59). This phage infects both naturally occurring *P. aeruginosa* and laboratory strains such as PAO. It exhibits pseudolysogeny and shares significant DNA homology with bacteriophage E79.

The plasmid Rms149 (19, 34) was isolated from strain RM2140 (75). (See Appendix for complete procedure.) This plasmid is Tra^- Mob^- and can only be transferred by transduction. It contains determinants for carbenicillin (Cb) and streptomycin (Sm) resistance.

Preparation of phage lysates. Cell-free lysates were prepared by the method of Miller and Ku (45). Briefly, RM2140 was grown to late

Table 1. Bacterial Strains and Plasmids

Strain or Plasmid	Genotype	Reference or Source
Bacterial Strains		
PAO1	Prototrophic	Miller & Ku, 1978
PAO227	*ilv*-226 *leu*-13 *lys*-12 *met*-28 *pro*-82 *trp*-6 *his*-4	B. Holloway
PAO515	*met*-9011 *ami*E200 *nal*A5	D. Hass
RM40	*met*-28 *lys*-12 *trp*C6 *pur*-600	Miller & Ku, 1978
RM2140	As for PAO1 but containing Rms149	Saye *et al.*, 1987
Plasmid		
Rms149	Cb^r Gm^r Sm^r Su^r Mob^- Tra^-	Hedges & Jacoby, 1980; Saye *et al.*,1987

mid-exponential phase (50 $Klett_{660}$ Units) in Luria-Bertani (LB) broth with shaking. One-half milliliter of this culture was added to 0.1 ml of a UT1 phage lysate diluted to 1 X 10^7 PFU/ml. Three milliliters lambda top agar was then added and the mixture poured on an LB plate. After overnight incubation at 37°C, the lambda top agar overlay was scraped off and placed in a 50 ml disposable type centrifuge tube containing 0.5 ml chloroform. After centrifuging for 10 min at 5,000 x g in a Sorvall GLC-2B bench-top centrifuge, the supernatant fluid was transferred to a fresh tube and its titer determined by plating in lambda top agar overlays using PAO1 as the indicator strain. This procedure was repeated a minimum of two times to ensure that transducing particles contained only DNA from the donor strain.

Transduction protocols.

(i) Laboratory grown host bacteria. Cells to be transduced were grown to mid-exponential phase in LB broth at 37°C. They were then centrifuged at 4,300 x g in a Sorvall RC-5B refrigerated centrifuge employing an SS34 rotor and suspended in 1 ml TNM buffer (0.01 M Tris, 0.15 M NaCl, 0.01 M $MgSO_4$; pH 7.4). This gave a viable count of approximately 1 X 10^8 CFU/ml. One-half milliliter of this suspension was combined with 0.5 ml of the desired phage lysate diluted to the appropriate MOI. When an F116 lysate was used, the mixture was incubated for 10 min at 37°C. For a UT1 phage lysate, incubation was carried out at 21°C for 10 min. Cells were then pelleted to remove unabsorbed phages, washed with TNM buffer, and finally suspended

in 0.5 ml TNM buffer. This mixture was then plated in duplicate on appropriate selective media and incubated for two days at 37°C. Two controls were also included. One consisted of cells with no added phage for determining spontaneous mutation frequencies. The other contained phage with no added cells to confirm that the lysate contained no culturable donor cells.

(ii) Preparation of lakewater samples and transduction of natural microbial communities. Approximately ten liters of water was obtained from Lake Jansee, a small freshwater lake in suburban Chicago, Illinois, USA. Each sample was centrifuged in a 250 ml sterile polypropylene centrifuge bottle at 5,800 x g for 30 min in a Sorvall RC-5B refrigerated (4°C) centrifuge employing a GSA rotor until the microbiota from the total volume had been concentrated into a single pellet. The pellet was suspended in 20 ml of lake water, and a viable count of 2 X 10^6 CFU/ml was determined by plating on 0.1X Yeast-Extract-Peptone-Glucose (YEPG) agar (58). One hundred forty-four colonies were replica plated on LB agar plates (45) containing either carbenicillin (700 μg/ml) or streptomycin (1000 μg/ml) to determine background resistance. Lakewater microorganisms concentrated in this manner were utilized for transduction experiments directly or were grown to mid-exponential phase (40 $Klett_{660}$ Units) in LB broth at 37°C before transduction was attempted.

For transductions utilizing natural lake water, 0.5 ml of the concentrated lakewater community sustained at approximately 10^6 CFU/ml was added

directly to 0.5 ml of appropriately diluted phage lysate (depending on the MOI used) prepared by growth on RM2140. The balance of the procedure was similar to that used for laboratory grown hosts, except that all incubations were carried out at 21°C.

Selective media. Presumptive Rms149-containing transductants were selected by plating on LB agar containing carbenicillin (700 μg/ml). The presence of Rms149 was verified genetically by scoring for Sm^r on LB agar containing streptomycin (1000 μg/ml). Chromosomal transductants were scored by plating on *Pseudomonas* Minimal Medium (PMM) containing 0.4% glucose supplemented with the appropriate amino acids at a concentration of 25 μg/ml (45).

Molecular techniques. Verification of the presence of plasmid Rms149 in presumptive transductants was achieved through gel electrophoresis. Plasmid DNA was isolated by rapid alkaline lysis (74) and digested with *Eco*R1 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) utilizing buffers and conditions recommended by the manufacturer. (See Appendix for complete procedure.) Samples were electrophoresed on 0.7% agarose gels. Digestion patterns were compared to those of plasmid DNA isolated from the parental RM2140 strain.

Results and Discussion

UT1-mediated transduction of laboratory strains. Before attempting to transduce natural lakewater isolates, it was necessary to first verify that UT1 was capable of transducing both plasmid and chromosomal DNA. Laboratory strains were utilized for this effort.

(i) Chromosomal transduction. Transduction of chromosomal DNA was carried out on three *P. aeruginosa* strains, RM40, PAO515, and PAO227. RM40 was transduced with a cell-free lysate prepared on PAO515 while the remaining two strains were transduced with a cell-free lysate prepared on PAO1. In each case, all alleles tested were found to be transducible. At a MOI of one, the *lys*, *met*, *trp*, and *pur* markers on the RM40 chromosome exhibited transduction frequencies from 1×10^{-10} to 3×10^{-10}. The *met* allele of PAO515 recombined at a frequency of 6×10^{-9} at a MOI of one while the *pro*, *met*, and *his* alleles of PAO227 exhibited recombination frequencies of approximately 2×10^{-8} when the MOI was 0.1 (Table 2). Spontaneous mutation frequencies were below the level of detection in these experiments. These transduction frequencies are similar to those previously reported for bacteriophage F116- and øDS1-mediated transduction (76).

All of the above chromosomal transduction frequencies were calculated at MOIs of 1 or 0.1. Differences in frequencies were observed when the multiplicity of infection was varied (Table 3). PAO515 and PAO227 were transduced with UT1 cell-free lysates prepared on PAO1 containing the

Table 2. Phage UT1-mediated transduction of chromosomal genes[1]

Donor	Recipient	Allele	MOI[2]	Transduction Frequency (Transductants/10^8 PFU)
PAO515	RM40	*lys-12*	1	0.03
		met-28	1	0.01
		trpC6	1	0.02
		pur-600	1	0.03
PAO1	PAO515	*met-9011*	1	0.6
PAO1	PAO227	*pro-82*	1	0.06
		pro-82	0.1	2.0
		met-28	0.1	3.0
		his-4	0.1	2.0

[1]Cell-free lysates prepared on the donor strain were used to transduce the recipient strain.

[2]MOI, multiplicity of infection; approximate MOIs were used.

Table 3. Affect of multiplicity of infection (MOI) on chromosomal transduction mediated by UT1

MOI[2]	Transduction Frequency (Transductants/10^8 PFU)[1]			
	met-9011	*met-28*	*his-4*	*pro-82*
10	0.08	ND[3]	ND	ND
1	0.6	ND	ND	0.06
0.1	50	3.0	2.0	2.0
0.01	900	10	220	ND

[1]PAO515 (*met-9011*) or PAO227 (*met-28*, *his-4*, *pro-82*) were transduced using a cell-free lysate prepared on RM2140. A representative experiment for each allele is reported.

[2]MOIs are approximate.

[3]ND, Not determined.

plasmid Rms149 at MOIs ranging from 0.01 to 10. In all cases, the transduction frequency increased dramatically as the MOI decreased. This is most likely due to the fact that the frequency of multiple infection in which a potential transductant is also infected with a lethal virion is reduced as the MOI decreases (43). Thus, the number of transductants surviving to be counted increases.

The transduction frequencies presented above indicate that non-adjacent chromosomal markers are being transduced by UT1 at roughly equal frequencies. This is characteristically associated with generalized transduction as opposed to specialized transduction, seen in phage λ, where only a restricted group of genes are transduced (28, 43). UT1 is therefore a generalized transducing phage, much like phages F116 and DS1 (47, 76).

(ii) Plasmid transduction. Transduction of plasmid DNA with UT1 was achieved by infecting *P. aeruginosa* strain PAO515 with a cell-free lysate of bacteriophage UT1 prepared on RM2140 (PAO1 containing the plasmid Rms149, which is a Tra^- Mob^- plasmid, thus insuring that conjugation could not occur). After infection, the transduction mixture was plated on LB agar plates containing carbenicillin (500 μg/ml). Resulting colonies were verified as transductants by DNA isolation and restriction endonuclease digestion analysis (Fig. 1). It is evident from these results that UT1 is an efficient transducer of plasmid DNA in laboratory strains (Table 4).

UT1-mediated transduction of a natural lakewater community. We wished to determine if generalized transducing phages of *P. aeruginosa*

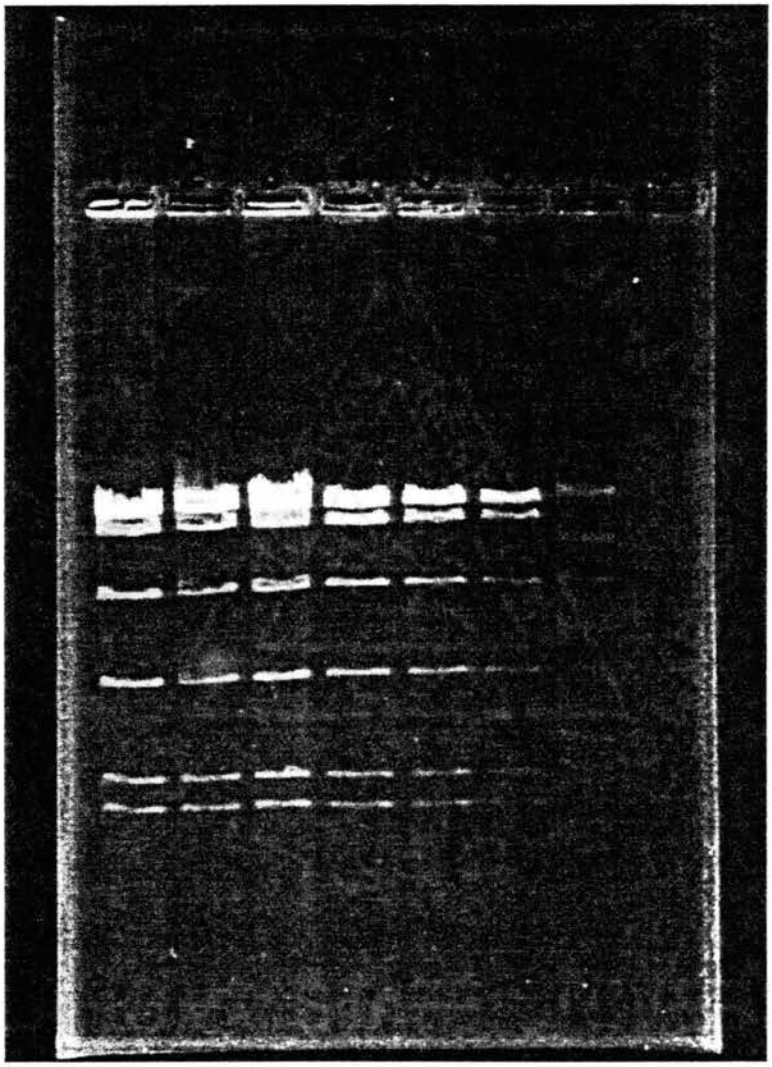


Fig. 1. Restriction patterns of plasmid DNA isolated from *P. aeruginosa* strain PAO515. Lanes: 1 to 5 *Eco*R1 digests of PAO515 transductants; 6, *Eco*R1 digest of plasmid Rms149 isolated from *P. aeruginosa* strain RM2140; 7, *Hind*III digest of lambda DNA; 8, empty.

Table 4. Plasmid transduction of PAO515 utilizing a UT1 lysate prepared on RM2140[1]

MOI[2]	pRms149 Transductants/10^6 PFU
1	0.97
0.1	9.1
0.01	64
0.001	380

[1] RM2140 is PAO1 containing pRms149

[2] MOIs are approximate.

could transduce members of a freshwater microbial community. For this study, we initially collected samples of water from Lake Jansee, a eutrophic lake in suburban Chicago, Illinois. A nine month analysis (October-June) of the microbiota of this lake revealed that the concentration of colony forming bacteria was maintained at 10^5 CFU/ml and that *P. aeruginosa* group organisms constituted 1-20% of the total CFU's for the entire study period (43). Phages capable of growing on *P. aeruginosa* laboratory strains were present in concentrations of 10^3-to-10^4 PFU/ml for the entire sampling period except in the winter months (February and March) when they fell to as little as 1.0 PFU/ml (43).

Bacterial cells concentrated from 10 l Lake Jansee water samples were used as hosts for UT1 mediated transduction at concentrations approaching 2 X 10^6 CFU/ml. A cell-free lysate of UT1 prepared on PAO1 containing the Rms149 plasmid was once again utilized as the transducing agent in order to demonstrate that plasmid DNA could be transferred to cells within a natural lakewater population.

Transduction experiments were performed under two different protocols. In the first, lakewater microorganisms concentrated from 10 l water samples were used directly without employing prior incubation or selective measures. Samples of the lakewater concentrate, maintained at approximately 10^6 CFU/ml, were added directly to suspensions of appropriately diluted phage lysate. All subsequent incubations were carried out at 21°C.

Under the second protocol, concentrated lakewater samples were incubated at 37°C in LB broth (45) prior to carrying out the transductions. It was our belief that this incubation protocol should have selectively favored growth of *P. aeruginosa*. *P. aeruginosa* is one of the few species of pseudomonads capable of growth at 37°C (22). Since preliminary experiments performed with laboratory strains of *P. aeruginosa* had demonstrated that UT1 could transduce *P. aeruginosa*, we hoped that selecting for this organism would give us an initially better chance of identifying transductants among the lakewater community.

Transductants were selected according to their resistance to streptomycin at 1000 μg/ml (Sm_{1000}) and carbenicillin at 700 μg/ml (Cb_{700}). Microorganisms naturally resistant to these concentrations of antibiotics were also present in the lake water communities, albeit at low levels; 0.016% of the total recoverable microbial population exhibited resistance to Cb_{700}. When 144 of these Cb^r colonies were examined, four (2.8%) were found to also be resistant to Sm_{1000}. When examined by restriction analysis, none of these organisms contained extrachromosomal DNA with a restriction pattern similar to Rms149. In any case, all transduction frequencies were corrected for the frequency of naturally occurring resistant organisms.

Transductants appearing in lakewater samples incubated at 37°C in LB broth occurred at frequencies of 10^{-4}-to-10^{-5}. Unexpectedly, prior incubation of lakewater samples at 37°C actually reduced transduction frequencies. This is likely due to adaption of natural populations of *P. aeruginosa* to their environ-

ment. Instead of selecting for *P. aeruginosa* present in lakewater communities, incubation at 37°C may have killed many potential recipients who had adapted to growth at lower environmental temperatures. When F116 phage lysates were used, similar results were obtained (Table 5). All presumptive transductants were shown to be resistant to both carbenicillin and streptomycin and to contain plasmid DNA with a restriction pattern identical to that of Rms149 (Fig. 2).

Conclusions

In this study, it has been demonstrated that a naturally occurring bacteriophage, UT1, is fully capable of acting as a generalized transducing vector capable of transferring both plasmid and chromosomal DNA. It has also been shown that this and other generalized transducing phages are capable of horizontal transfer of plasmid DNA to natural microbial assemblages collected from lakewater habitats. These are important observations because they are the first demonstrations of transduction of a naturally occurring host mediated by a naturally occurring bacteriophage. While these studies do not address the kinetics of gene transfer in the aquatic environment, they demonstrate that model systems based on naturally occurring elements can be adapted to study the consequences of bacteriophage-mediated horizontal gene transfer on the evolution and genetic diversity of microbial populations present in aquatic habitats. Such studies will provide important insights into the biological and genetic ecologies of these environments and will be useful in identifying and defining the effects and potential risks associated with the deliberate introduc-

Table 5. Plasmid transduction of natural lakewater communities

Recipient	MOI	Experiment Number[1]	Transductants/10^6 PFU			
			F116		UT1	
			Treatment of Recipient		Treatment of Recipient	
			None	37°C	None	37°C
PAO515	0.1	1	ND[2]	10	ND	10
Lake	0.1	1	9000	1500	2000	62
Jansee	0.05	2	2000	220	2000	120
	0.2	3	ND	580	ND	59

[1]Experiment No. indicates that the data present are from independent experiments conducted on lakewater samples collected at different times.

[2]ND = Not determined. PAO515 is a well characterized laboratory strain of *P. aeruginosa* and served as a control for the environmental samples.

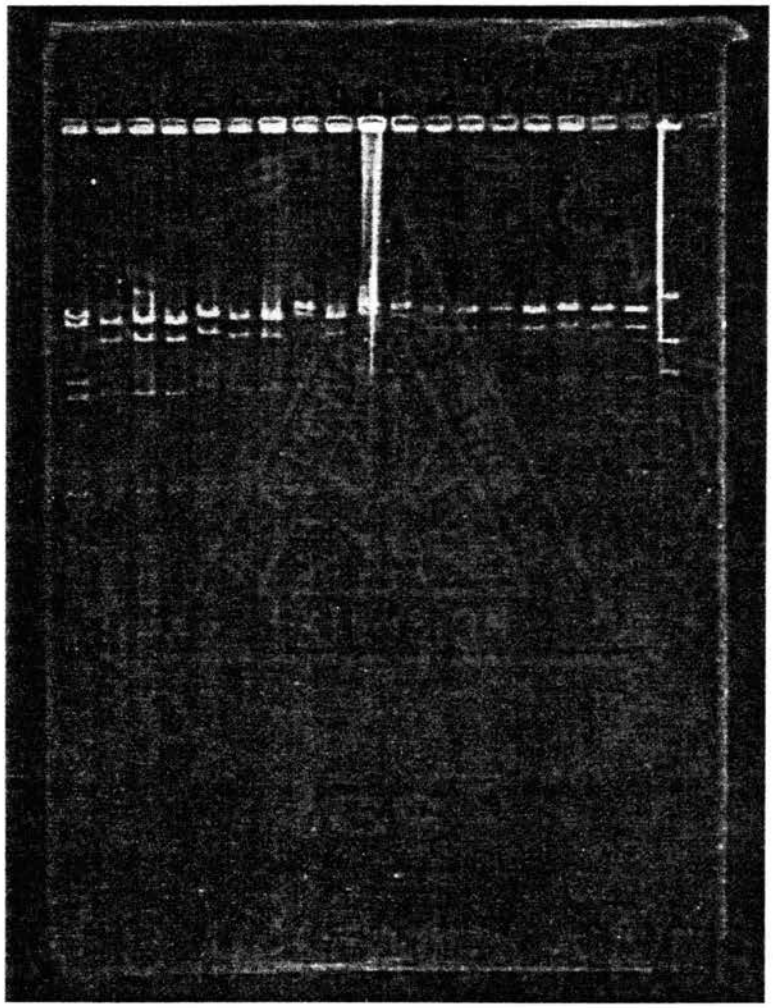


Fig. 2. Restriction patterns of plasmid DNA isolated from lakewater transductants. Lanes: 1 to 17, *Eco*R1 digests of lakewater transductants; 18, *Eco*R1 digest of plasmid Rms149 isolated from *P. aeruginosa* strain RM2140; 19, *Hind*III digest of lambda DNA. Lanes 1, 8, 10, and 11 were common patterns associated with partially cut Rms149.

tion of new genetic elements into these environments through biotechnological practices.

CHAPTER IV

CHARACTERIZATION OF PSEUDOLYSOGENIC RELATIONSHIPS BETWEEN A VIRULENT BACTERIOPHAGE AND A NATURAL FRESHWATER MICROBIAL COMMUNITY

Introduction

Recent studies have documented extensive reservoirs of viral particles in environmental ecosystems (6, 9, 20, 63, 64, 87). However, our current knowledge of microbial and viral population dynamics does not support the production nor maintenance of large viral populations within natural environments. Our studies of natural phage-host interactions consists of observations made under starvation conditions typically present in natural habitats. Under such circumstances, phage replication has been shown to be significantly altered, with latency periods being lengthened, burst sizes being reduced, and the overall virulence of lytic viruses diminished (32, 59). In addition, the infective half-lives of virions under natural conditions has been shown to be less than 48 hours in most circumstances (59). Consequently, viral populations should remain fairly small. In actuality, they do not.

We have hypothesized that these large viral populations are due to a highly neglected phenomenon called pseudolysogeny. Pseudolysogeny was first described by Twort (85) in 1915 but never actually studied until 1961 when

Romig and Brodetsky (72) used it to describe the relationship between various soil bacilli and their viruses. Pseudolysogeny describes a bacteriophage-host cell interaction in which the nucleic acid of the phage, upon infection of an appropriate host cell, neither establishes a long-term, stable relationship (i.e., lysogeny) nor elicits a lytic response. Rather, the phage nucleic acid simply resides within the cell in a non-active state of limbo. It is our hypothesis that due to the cell's highly starved condition, there is insufficient energy available for the phage to initiate either of these typical laboratory responses to infection. However, upon nutrient accruement by the host cell, the phage acquires the necessary energy to allow gene expression, leading to either the establishment of a state of true lysogeny or replication and expression of the viral genome leading to virion formation and lysis of the host cell.

In this chapter is presented a study of the pseudolysogenic response of bacteriophage UT1, both under laboratory and environmental conditions. Our studies suggest that pseudolysogeny may increase the effective environmental life of phage genomes well beyond the active infectious half-lives of their virions. Such an increase in genome survival may, in part, provide an explanation for the large environmental reservoirs of bacterial viruses commonly being observed.

Materials and Methods

Bacterial strains and bacteriophages. *P. aeruginosa* strains PAO1 (prototrophic) (45) and PAO303 (*argB21*) (29) were used in this study. Phage UT1 was isolated from a natural freshwater source (59). It exhibits

virulence under laboratory conditions, but appears pseudolysogenic under natural conditions (32). It is also a generalized transducing phage (71).

Media. Luria-Bertani (LB) agar plates were routinely used as a source of nonselective medium (45). Selective media consisted of *Pseudomonas* Minimal Medium (PMM) containing 0.4% glucose supplemented with arginine at 25 μg/ml (45).

Field site. Lakewater sampling and *in situ* incubations were performed at Lake Sanborn, a small (5 ha), semi-oligotrophic freshwater lake near Stillwater, OK with a mean depth extending to approximately 2 m and very low abiogenic turbidity (18). Phosphate has been shown to be the limiting nutrient in this environment (18, 57). Temperature, pH, redox values, and relevant weather conditions were obtained at the time of each sampling.

Enumeration of bacteriophages by transmission electron microscopy (TEM). Phage enumeration from freshwater sources was performed as described by Bratbak and Heldal (9). Briefly, 100 ml samples of lake water were preserved with 2% electron-microscope-grade formaldehyde immediately after sampling. Carbon-coated formvar grids (400 mesh) were taped onto a cellulose nitrate filter cut to fit within a Nalgene 38.5 ml open-top, thick-wall polyallomer ultracentrifuge tube with a flat bottom molded in epoxy glue. Samples were centrifuged at 12°C in a Beckman SW28 swinging bucket rotor at 100,000 x g for 2.5 hours after which the supernatant fluid was discard-

ed and the grids gently removed. Grids were stained with 2% uranyl acetate for 30 sec followed by a single rinse with sterile distilled water. After drying, the grids were examined in a JEOL Temscan 100CX transmission electron microscope at a magnification of 100,000 X using condenser aperature number one and objective aperature number three.

The final concentration of particles in the sample was calculated using the following equations (9):

The area of the view field (A, in centimeters squared) is first calculated by:

$$A = \pi \left(\frac{r_{\text{view field}}}{\text{mag}} \right)^2$$

where $\pi = 3.14$, $r_{\text{view field}}$ is the radius of the microscope's view field (in centimeters), and mag is the magnification. The water volume from which particles are harvested during centrifugation (V, in milliliters) can then be calculated by:

$$V = \frac{A\left(R^2 - r^2\right)}{2R}$$

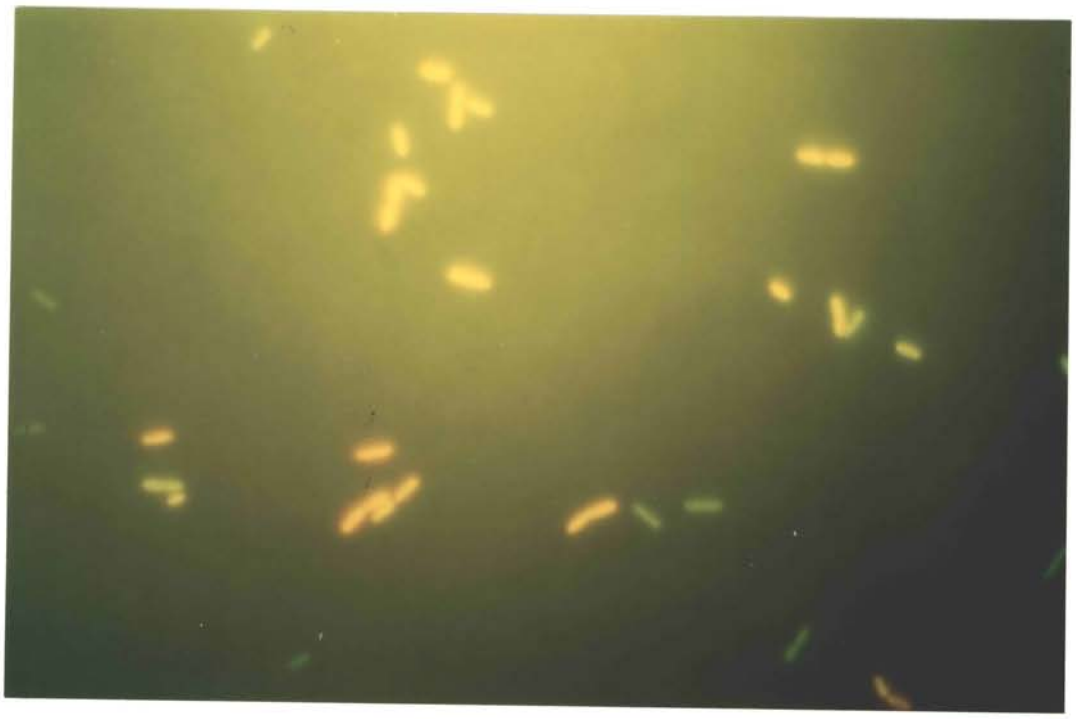
where R is the maximum and r the minimum radius (in centimeters) of the water in the centrifuge tube during centrifugation (according to rotor used). The concentration of particles in the sample per milliliter is then determined by dividing the average particle count per view field by V.

Microcosms.

(i) Batch. Various experiments required progressive starvation of cells. This was accomplished by the use of batch cultures which consisted of 2 l Erlenmeyer flasks containing a variation of PMM in which the sodium citrate had been omitted (PMM-c). Yeast extract was provided as an initial nutrient source at a final concentration of 1 x 10^{-5}% (67). Initial inoculants were grown overnight in LB broth at 37°C, diluted 1/100 in PMM-c supplemented with 0.5% yeast extract and grown to mid-exponential phase at 37°C. These cultures were then washed twice with PMM-c (containing no yeast extract) and inoculated into the microcosm at the desired concentration. Incubations occurred at room temperature with slow shaking (50 rpm). Some microcosms were periodically spiked with yeast extract at a final concentration of 1 x 10^{-5}% to emulate random influxes of nutrients as might be expected in natural aquatic environments. Acridine-orange-direct-counts (AODC), where starved cells fluoresce green and actively growing cells fluoresce red, were routinely performed to ensure that adequate cellular starvation was occurring (Fig. 3). (See Appendix for complete procedure.)

(ii) Chemostat. New Brunswick Bioflo Model C30 bench top chemostats (New Brunswick Scientific Co.) were utilized in these experiments. Chemostats consisted of a 2 l culture vessel and a 10 l medium reservoir containing PMM-c. Yeast extract was provided as the sole nutrient source at a final concentration of 1 x 10^{-5}%. Incubations occurred at room temperature at an agitation rate of 200 rpm. The culture vessel was inoculated with PAO303

A.



B.

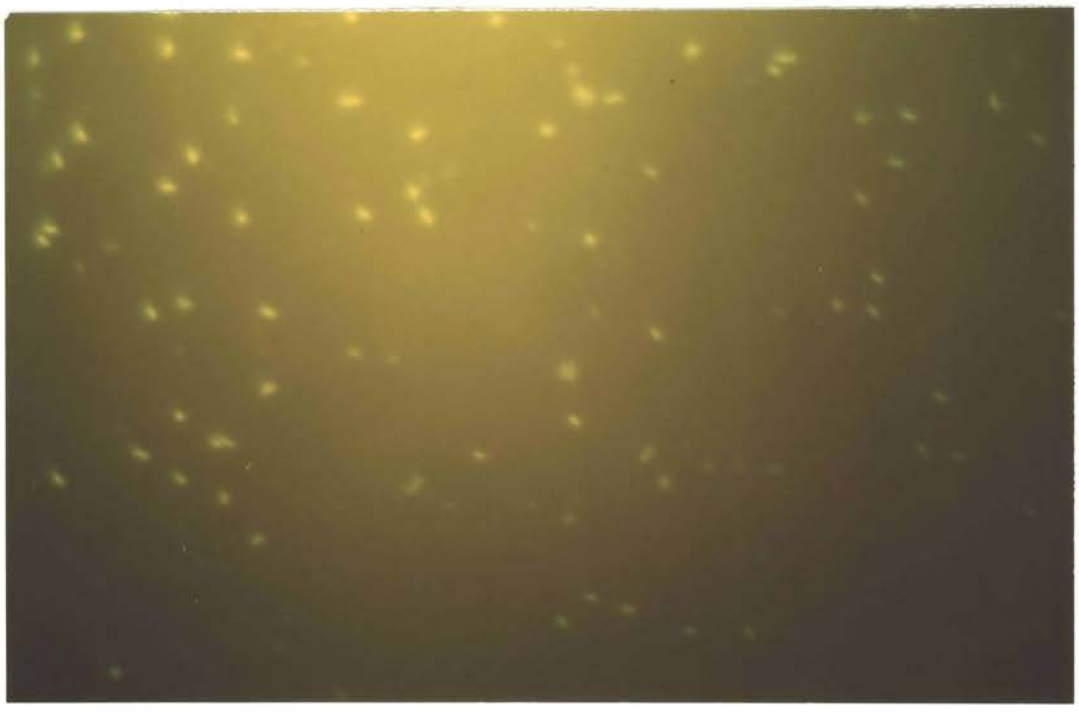


Fig. 3. Acridine-orange direct counts (AODC) of a batch microcosm containing *P. aeruginosa* strain PAO303 after (A) 3 days, and (B) 16 days of incubation. The actively growing cells in (A) fluoresce red while the starved cells in (B) fluoresce green. The severe decrease in size of starved cells can also be seen.

and phage UT1 at an MOI of one (approximately 1 x 10^6 CFU/ml and 1 x 10^6 PFU/ml). Inoculants were prepared as for the batch microcosms.

(iii) *In situ* incubated. Microcosms incubated *in situ* were prepared as described by Saye *et al.* (76). One-liter Lifecell tissue culture chambers (Fenwal Laboratories, Deerfield, IL) were filled with 500 ml filter-sterilized (0.2 μm) lake water. Preparation and inoculation of strain PAO303 and bacteriophage UT1 were identical to that of the chemostat microcosms. The chambers were incubated *in situ* in Lake Sanborn either on the surface or at a depth of 2 m. Some microcosms received periodic nutrient spikes of yeast extract at a final concentration of 1 x 10^{-5}%.

Bacteriophage and infective center enumerations. Bacteriophage counts were determined by filtering samples through 0.45 μm syringe filters to remove bacterial cells. The remaining phages were then diluted and 0.1 ml of the desired dilution combined with 0.1 ml of PAO1 (in mid-exponential phase) and 2.5 ml λ top agar (45). This mixture was poured onto LB agar plates and the resulting plaques were counted following overnight incubation at 37°C.

Infective centers refer to bacteria which are in the latent period of lytic infection (32). Enumeration of infective centers was carried out in the same manner as bacteriophage counts except that no initial filtration was performed. The apparent infective center plaque counts obtained from these plates represent the total number of both infective centers and free virions present in

the sample. By subtracting the titer of free phages obtained from the corresponding apparent infective center count, we obtained a number representing the actual concentration of infective centers (32).

Identification of phage-releasing isolates. PAO303 cells were sampled from batch or lakewater microcosms at various times and routinely examined to determine what percentage were activated to release phage virions. One-hundred colonies per sample were replica-plated onto a top agar overlay of PAO1 and incubated overnight at 37°C. A zone of lysis surrounding a colony was indicative of the spontaneous release of virions (Fig. 4).

Colony hybridizations. Probes were constructed by isolating UT1 DNA according to Silhavy *et al.* (78) and labeling with a Genius Nonradioactive Labeling Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). (See Appendix for complete procedure.) Colony hybridizations were performed to determine the frequency of cells harboring phage DNA (preprophages). They were carried out as outlined in the Genius protocol (1) using Magnagraph 0.45 μm (mean pore size), 85 mm diameter nylon membranes (Micron Separations, Westboro, MA).

Time of nutrient spiking experiment. We wished to determine whether the timing of the nutrient spike had any affect on phage interaction with a starved host population. Submicrocosms were routinely removed from a main batch microcosm of PAO303 inoculated at 1 x 10^6 CFU/ml. To each

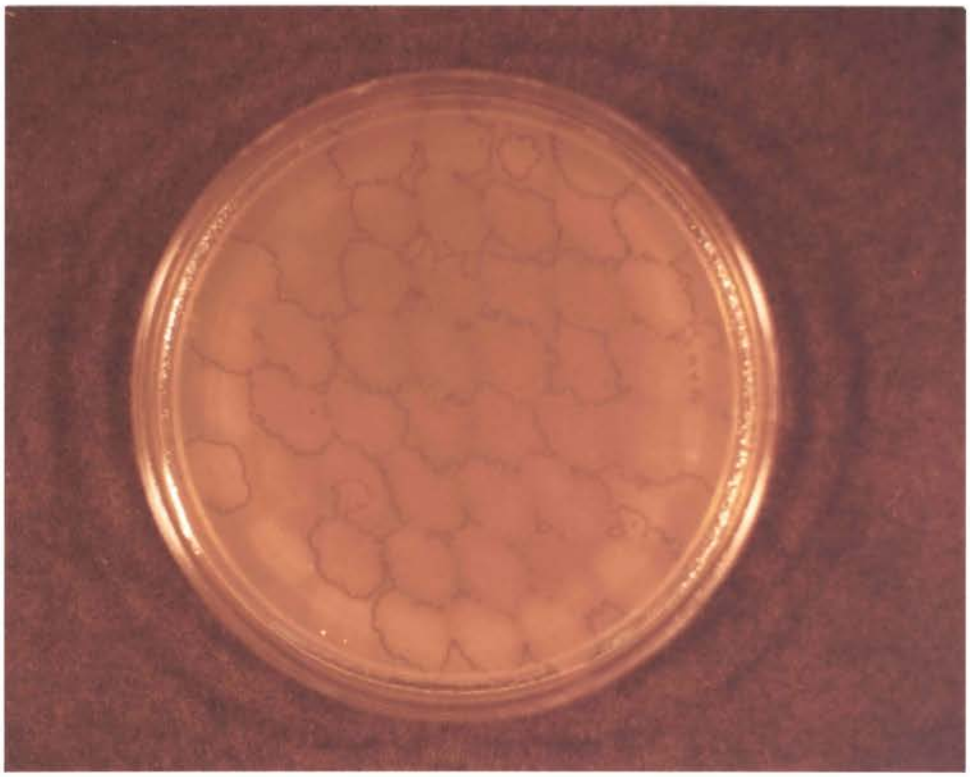


Fig. 4. Colonies of *P. aeruginosa* strain PAO303 replica plated onto a lawn of PAO1. Zones of lysis surrounding the colonies indicate that cells are releasing phage.

submicrocosm, phage UT1 was added at 1 x 10^7 PFU/ml, allowed to infect for 8 hours, and then removed by centrifugation (4,000 x g, 12 min). Addition of phage was followed by an immediate nutrient spike, by a spike after 24 hours, or by no spike whatsoever.

Results and Discussion

The most important environmental factor affecting a natural microbial population is the availability of energy, and, since most ecosystems are oligotrophic, energy resources are most often limiting (53). As a result, most bacteria in any given ecosystem are subsisting in a starved state, and are essentially inactive. Roszak and Colwell (73) have referred to such cells as viable but nonculturable. In these cells basal metabolism is reduced to the lowest level of subsistence and growth in any form is eliminated. Growth only occurs as blooms following the sporadic addition of energy to the ecosystem from some external source. It has been suggested that this is the conventional state of bacterial populations in environmental ecosystems (73).

Relationship between degree of starvation and bacteriophage production. We wished to investigate whether it was possible for bacteria in a starved state to interact with bacteriophages in a productive manner. We began our studies by evaluating the effects of long-term starvation on virion production. Batch cultures were inoculated with PAO303 at 4 x 10^6 CFU/ml and phage UT1 at 1 x 10^5 PFU/ml and incubated for approximately 80 days. Some cultures were periodically spiked with nutrient while others were

not spiked. This protocol simulated the feast-famine conditions which bacteria in freshwater environments typically encounter. Virion production and lysogeny establishment were found to be directly related to nutrient addition (Fig. 5). The control microcosm, held under strict starvation conditions, exhibited no substantial increases in the frequency of phages or phage-releasing clones within the population of bacteria. However, following nutrient addition, an identical culture showed pronounced increases within 12 hours after each spike. The lack of phage production in the unspiked culture was not due to the inability of cells to become infected since the frequency of phage-resistant cells remained undetectable during the entire incubation period. PBR increases occurred only after nutrient addition and then slightly decreased due to the short half-life (18 hours) of the phages under these conditions (59). The overall increase in the PBR was due to a decrease in cell numbers (Fig. 6). However, individual PBR increases corresponded to elevated phage production following nutrient spikes. These data illustrate that nutrient availability dramatically affects phage-host interactions.

Effect of the time of nutrient spiking on starved cultures. If our hypothesis concerning the pseudolysogenic state is correct, phages should be inducible by nutrient addition only when they are given the opportunity to first infect and maintain themselves within the starved cell. A phage infecting a well-fed cell should only exhibit lytic growth. This was shown to be true when phage UT1 was added to PAO303 submicrocosms followed by an immediate nutrient spike or a spike after 24 hours. Submicrocosms exhibited greatest

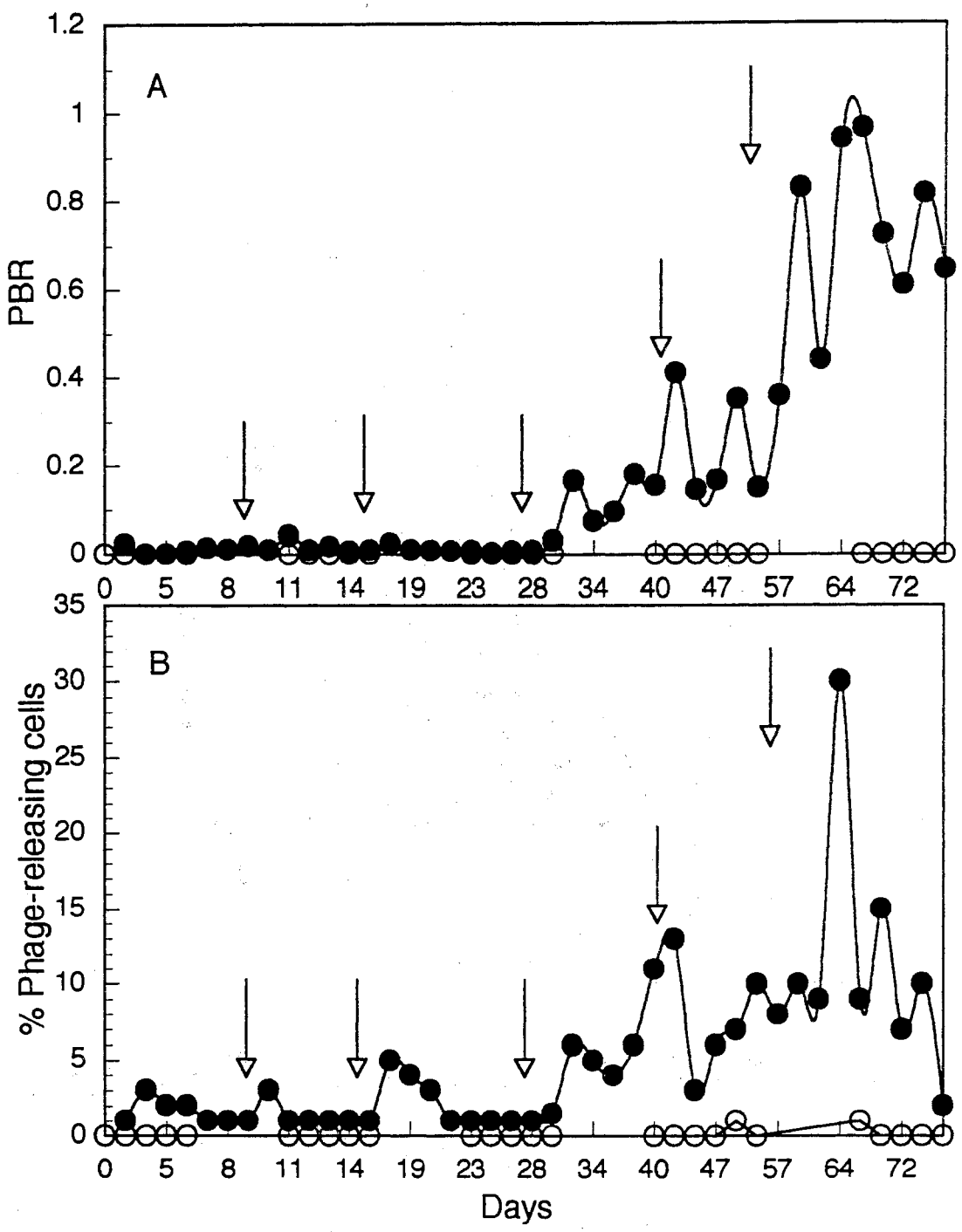


Fig. 5. (A) Phage-to-bacterium ratio (PBR) and (B) percent of cells releasing phage in a microcosm containing PMM-c and yeast extract at 1 x 10^{-5}%. Microcosms were inoculated with PAO303 and phage UT1 and spiked with nutrient (1 x 10^{-5}% yeast extract) at various intervals. (O) No nutrient added, (●) Nutrient added, (↓) Time of nutrient addition.

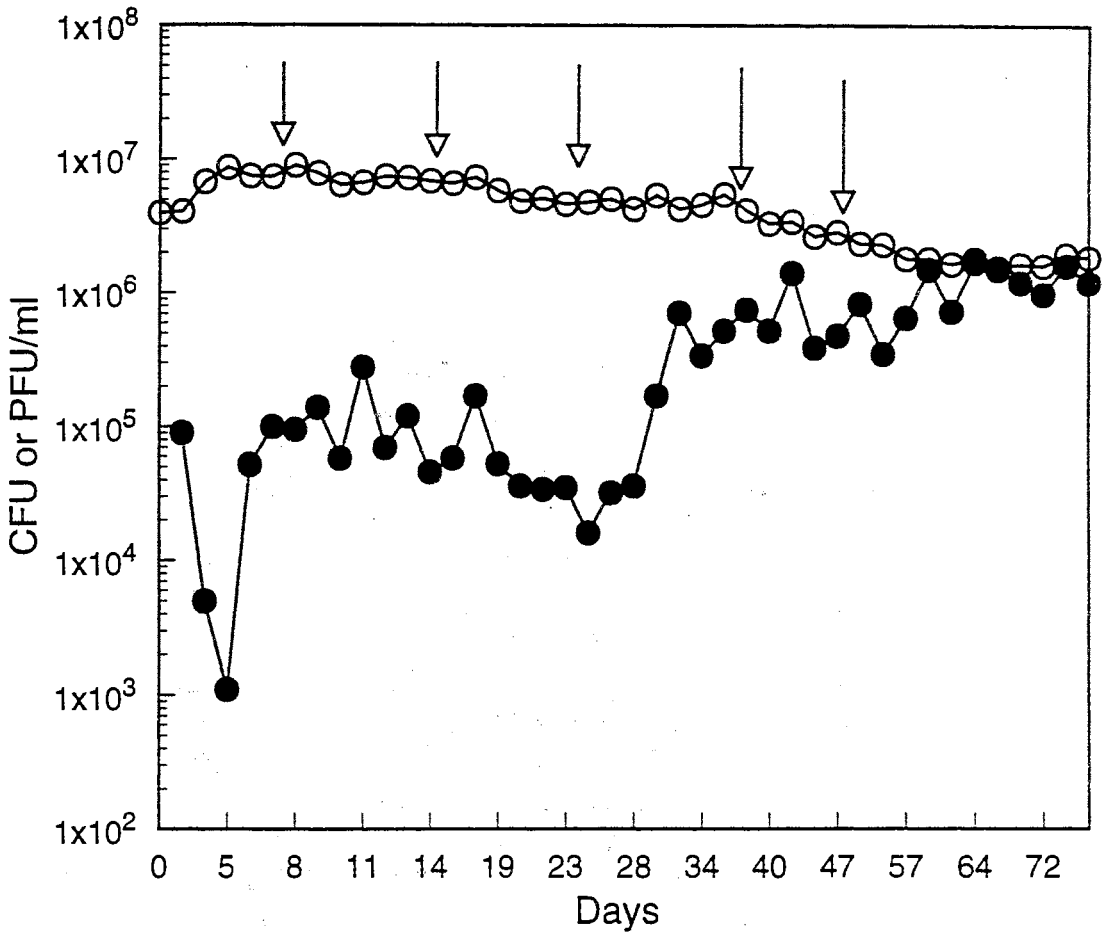


Fig. 6. Long-term starvation of a batch culture inoculated with *P. aeruginosa* strain PAO303 (O) and bacteriophage UT1 (●). Nutrient spikes of yeast extract (1 x 10^{-5}% final concentration) were added at times indicated by arrows.

virion production and frequency of phage-releasing clones when nutrient spiking occurred 24 hours after phage inoculation (Fig. 7). Infection with concurrent nutrient addition led only to low levels of virion production, presumably because the phage could only sustain itself lytically in cells that simultaneously received nutrients. This is similar to what is seen when phage UT1 is grown under laboratory conditions using well-fed cells. In such a case, the virulent phenotype is habitually observed (32, 59).

Chemostat microcosms. Our first clear indication of the occurrence of a pseudolysogenic response was observed under continuous culture conditions. Chemostats containing strain PAO303 and phage UT1 were incubated in PMM-c for one month at a generation time (turn-over time) of 10 hours. The total fraction of cells containing phage genomes (tested positive in colony hybridizations when probed with UT1 DNA - Fig. 8) was compared to the fraction spontaneously releasing phage virions. We took the difference in these two numbers to represent the number of bacteria which contain a viral genome in the pseudolysogenic state (Table 6). Those releasing phage had most likely already undergone activation due to the added nutrient available to the host during growth of the colony on the plate. At all timepoints sampled, more colonies were shown to contain UT1 DNA than were actively releasing phage virions.

Unstable propagation of preprophage. If pseudolysogens did, in fact, contain phage genomes in which the pseudoprophage had not yet been

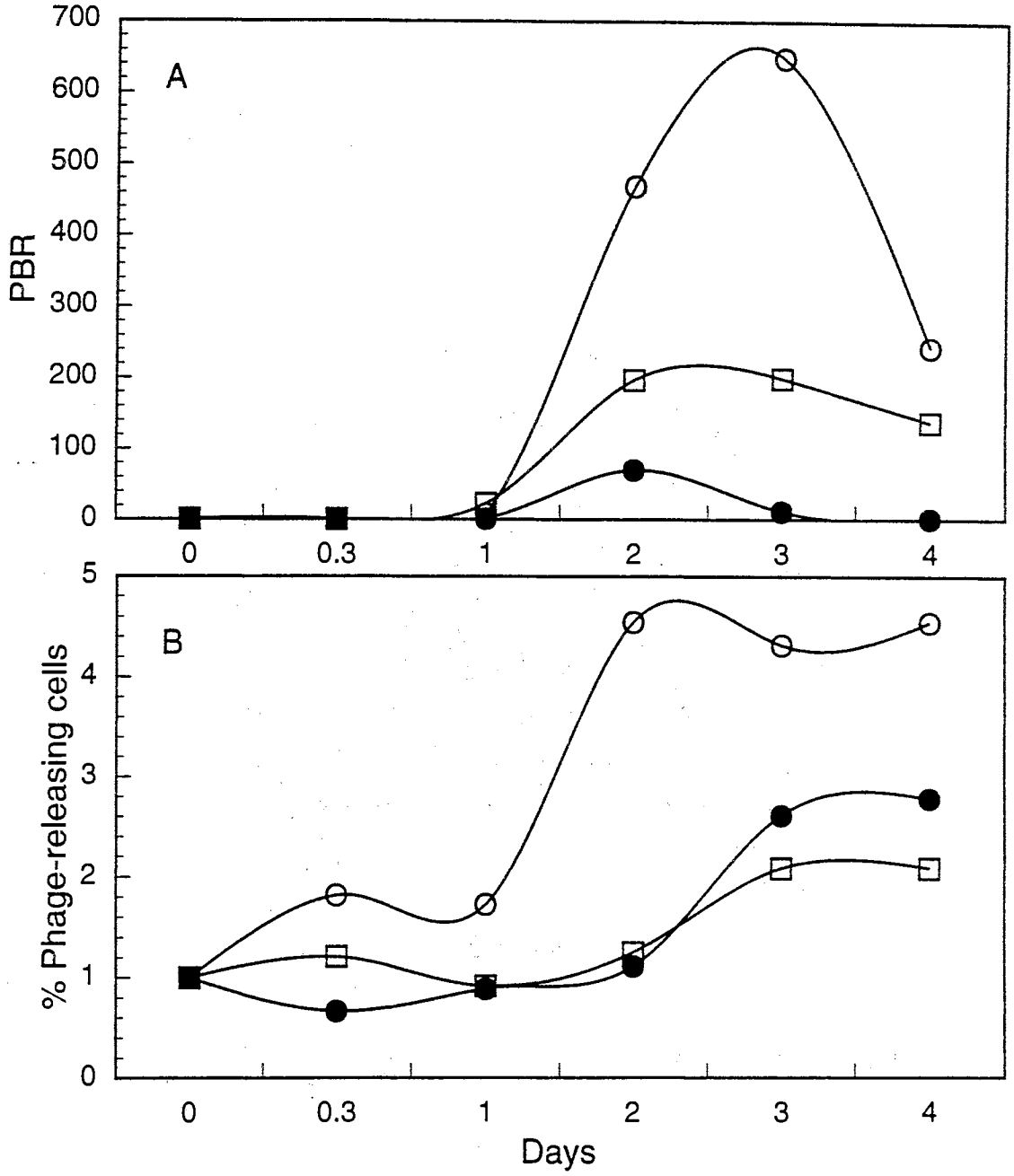


Fig. 7. Submicrocosms removed from a main batch culture inoculated with PAO303 and incubated for 25 days. (A) Phage-to-bacterium ratio (PBR); (B) Percentage of cells actively releasing phage. (All values divided by T_0.) (O) Phage UT1 was added 24 h after submicrocosm was removed, allowed to infect for 6 h, and then removed by centrifugation. Nutrient (1×10^{-5}% yeast extract) was added 24 h later. (●) Phage UT1 was added and removed 6 h later as explained above. No nutrient addition occurred. (□) Phage UT1 was added and removed 6 h later as explained above. Nutrient was added simultaneous to phage addition

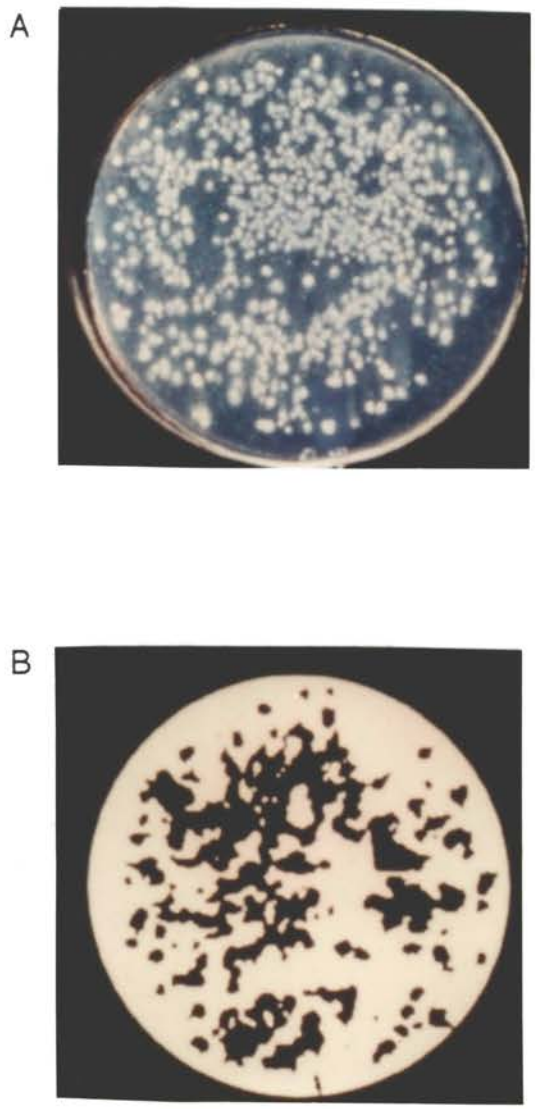


Fig. 8. Colony hybridization of a sample taken from a chemostat culture containing *P. aeruginosa* strain PAO303 and bacteriophage UT1. (A) The original plate on which the sample was isolated. (B) The nylon membrane onto which the colonies were transferred and hybridized with phage UT1 DNA.

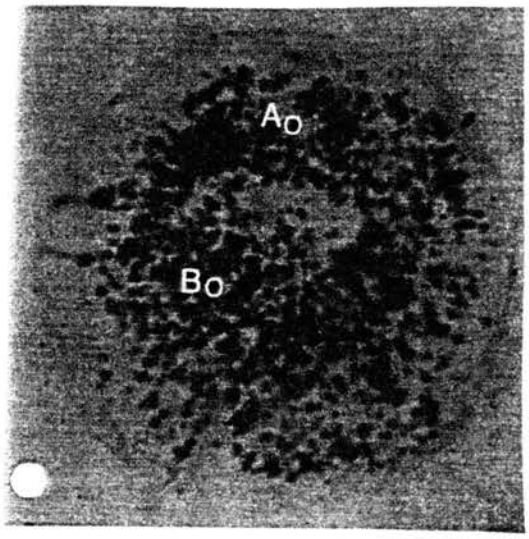
Table 6. Microcosm exhibiting the prevalence of cells that contain phage UT1 DNA but do not actively release phage (pseudolysogens) in an ideally starved chemostat culture. Data is presented as the fraction of cells that contain the phage genome from which is derived the percent of colonies actually shown to be releasing phage and the percent of colonies not releasing phage but shown to contain phage DNA via colony hybridization.

Day	Fraction of cells containing phage genome	% activated*	% not activated*
0	0/200	0	0
3	1/49	0	100
9	32/164	17	83
16	31/120	32	68
24	25/122	35	65

*Percent of those cells which contain the phage genome that are either activated or not activated to release phage

activated to replicate, one would predict that the majority of cells in a pseudolysogenic clone would not contain phage genomes at all. Like abortive transduction, the pseudoprophage would be segregated into only one daughter cell without replicating (2). To test this hypothesis, pseudolysogenic colonies from the chemostat samples were streaked onto LB agar plates and the resulting individual colonies were probed with UT1 DNA. Results indicated that only a small fraction of cells within a pseudolysogenic colony contained phage DNA (Fig. 9). The preprophage seemed to undergo haphazard transfer during cellular division. These data indicate that the phage nucleic acid had stabilized itself in the host cell in a form that does not allow fidelity of scheduled DNA replication coordinate with host genome replication and cell division.

Phage enumeration from a freshwater source. Previous studies have reported phage abundances in rivers and ocean waters to range from 10^3-to-10^8 particles/ml (6, 20, 63, 87). We wished to validate the presence of significant phage populations at our freshwater-lake field site, a small (5 ha) oligotrophic natural lake. Samples of lake water were prepared for phage enumerations as described above, and phage particles from each sample were counted within 100 TEM view fields (Fig. 10). An average of 4.1 ($\pm$ 0.4) x 10^6 virus particles/ml of lake water were observed. These data are consistent with the work of Replicon *et al.* (67) who observed concentrations of 1 x 10^4 PFU exclusively infective for *P. aeruginosa* per milliliter in a freshwater lake near Chicago, Illinois. PBRs in these samples were observed to be as high as 50:1, implying that most of the bacteria in the environment were infected with



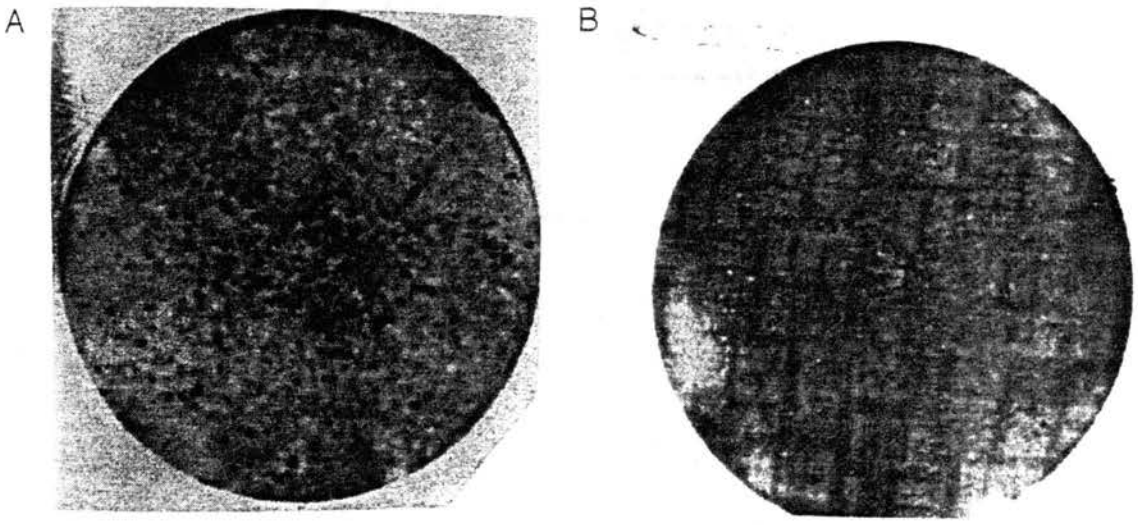


Fig. 9. Example of the unstable propagation of preprophages. Positively-hybridized colonies labeled (A) and (B) were removed from the original plate and each restreaked onto separate plates and then colony hybridized. One of the colonies contained UT1 DNA and produced positively-hybridized clones (A). The other produced no hybridization-positive clones and thus contained no UT1 DNA (B).

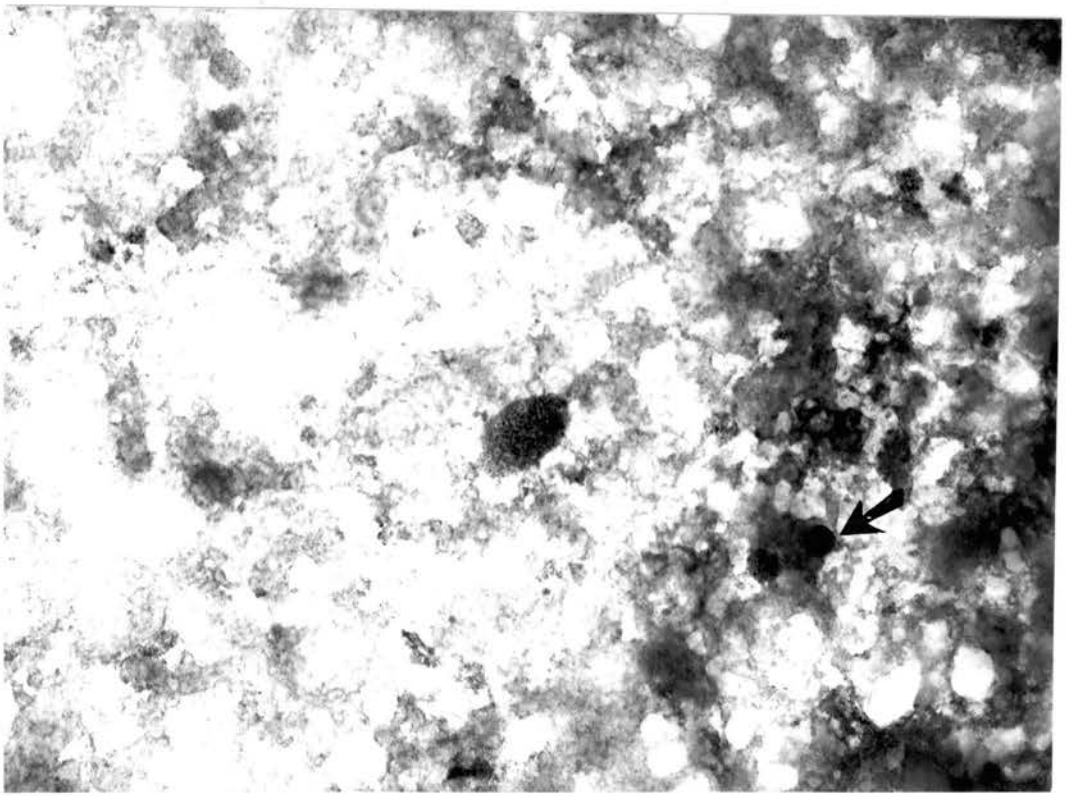


Fig. 10. Representative micrograph of lakewater samples. Viral particles are distinguished by their small size and dense staining due to the presence of nucleic acid in their head structure. 60,000X magnification

phages. Proctor and Fuhrman (64) used electron microscopy to estimate levels of phage infection in a marine environment. They concluded that as much as 70% of the cells in ocean waters were infected with phages at any one time. More recently, Suttle (82) estimated that up to 50% of the marine microbial population must be lysed per day in order to maintain bacteriophage levels observed in the marine environment.

***In situ* incubated microcosms.** In order to study pseudolysogeny in a condition closer to a true environmental situation, we incubated microcosms *in situ*. Filter-sterilized water from Lake Sanborn was used to prepare four microcosms, each inoculated with PAO303 and phage UT1. These were incubated in the lake either on the surface or at a depth of 2 m. Yeast extract was periodically added to half of the microcosms.

Microcosms displayed high concentrations of cells containing UT1 DNA, not all of which actively released phage virions (Table 7). Those not activated were considered to be in a pseudolysogenic state. The addition of nutrient did not produce the expected sharp increases in PBR numbers as was exhibited in the *in vitro* incubated microcosms (Fig. 6). This was probably due to the fact that nutrients were not limited from the *in situ* lakewater environments such that the small addition of yeast extract had little or no measurable effect.

Table 7. Comparison of the percent of phage-releasing cells (% activated) to pseudolysogenic cells (% not activated) in *in situ* incubated microcosms residing on the lake surface or at a depth of 2 m. The first column of each set lists the ratio of cells found to contain the phage genome. The second column represents the percent of cells actively releasing phage. The percent of pseudolysogens, those cells not activated to release phage but shown to contain phage DNA, is recorded in the third column of each set.

Day	Surface						Bottom					
	Chamber 1			Chamber 2			Chamber 3			Chamber 4		
	Fraction of cells containing phage genome	% activated*	% not activated*	Fraction of cells containing phage genome	% activated	% not activated	Fraction of cells containing phage genome	% activated	% not activated	Fraction of cells containing phage genome	% activated	% not activated
0	0/170	0	0	0/108	0	0	0/310	0	0	0/198	0	0
9	6/310	0	100	0/140	0	0	0/220	0	0	6/430	0	100
15	247/570	51	49	187/740	92	8	65/140	74	26	30/720	25	75
21	350/570	92	8	360/420	74	26	280/520	94	6	40/370	100	0
27	300/480	100	0	15/109	64	36	244/280	100	0	223/740	80	20
29	490/650	93	7	310/440	93	7	224/610	81	19	153/300	78	22
43	64/86	84	16	112/112	82	18	236/400	92	8	68/83	93	7

*Percent of those cells which contain the phage genome that are either activated or not activated to release phage

Conclusions

In this study, we have shown that pseudolysogenic associations between bacteriophages and their hosts have the potential to affect natural bacteriophage ecology dramatically. As a result of sequestering of phage genomes in the pseudolysogenic state, effective phage half-lives are greatly increased leading to the long-term survival of viruses that would otherwise be eliminated from the environment. Pseudolysogeny is likely to be an important factor in the maintenance of the large phage populations observed in aquatic environments, especially those that appear virulent under laboratory conditions.

Bacteriophages affect microbial ecosystems in at least two potentially significant ways. First, they act as bacterial predators, influencing various environmental food webs (64). Second, previous studies from our laboratory have shown that transduction, the viral-mediated horizontal transfer of genetic material, is a significant gene exchange mechanism among bacteria in natural aquatic ecosystems, capable of influencing genetic diversity and evolution (67, 71, 76). Transduction potentially plays a substantial role in the estimation of the efficacy and associated risks of applying genetically engineered microorganisms to natural environments. The potential of virulent bacteriophages to form pseudolysogenic relationships must be considered in estimating exposure of natural populations to introduced genetic sequences. Genetic material contained in preprophage genomes will have a longer half-life in the environment than will infective virions of the virus. Thus, estimates of exposure duration must be adjusted to account for the pseudolysogenic phenomenon.

Further studies will allow for the necessary development of predictive models of true phage-host interactions in oligotrophic environmental ecosystems.

CHAPTER V

CHARACTERIZATION OF PSEUDOLYSOGENIC RELATIONSHIPS BETWEEN A TEMPERATE BACTERIOPHAGE AND A NATURAL FRESHWATER MICROBIAL COMMUNITY

Introduction

During the last two decades, the potential for virus-mediated horizontal transfer of genetic material (transduction) has been recognized as a potentially significant gene exchange mechanism among bacteria in aquatic habitats (4, 54, 69, 75, 76). Both plasmid and chromosomal DNA can be transferred among bacteria in these environments. In addition, the occurrence of transduction among bacteria has been shown to act to maintain novel phenotypes in a bacterial population that would otherwise be eliminated from the gene pool if horizontal gene transfer did not occur (67).

However, transduction has only recently been considered as an important environmental phenomenon because numbers of bacterial viruses in environmental ecosystems were regarded as insignificant until, in 1989, Oivind Bergh and co-workers at the University of Bergen in Norway reported viral populations in ocean waters to be three to seven times higher than what was previously assumed (6). Observations of samples using transmission electron microscopy revealed as many as 1×10^8 bacterial viruses per milliliter of water.

However, our current knowledge of viral behaviors in environmental ecosystems does not support such high numbers. We have been investigating potential mechanisms that would increase the infective half-lives of viruses in nature. Such changes would increase viral reservoirs and lead to higher numbers of phage particles being observed. We have previously demonstrated that a process referred to as pseudolysogeny occurs in nature among viruses that only demonstrate lytic growth patterns in the laboratory. Pseudolysogeny may, in part, be responsible for maintaining high-density viral populations in the environment.

Pseudolysogeny describes a phage-host interaction in which the phage, upon infecting it's host, does not initiate either a lysogenic or lytic response, but rather simply exists within the cell in an unstable, non-active form (3). This response occurs because the host cell is starved and cannot provide the phage with the necessary energy required for it's expression. Cellular starvation in environmental ecosystems is commonplace. When a nutrient source is provided, however, the phage acquires it's essential energy and is activated to initiate virion formation and subsequent cell lysis. The overall result of pseudolysogeny is an extension of the viral genome's half-life, which may, perhaps, explain the occurrence of large environmental phage populations.

In this chapter, the goal was to determine whether viruses such as phage F116 that establish true lysogeny in their hosts under laboratory conditions would be able to do so in the environment. Alternatively, these

viruses might also be forced into the pseudolysogenic state due to the starved condition of the host cell they infected.

Materials and Methods

Bacterial strains and bacteriophages. Batch and chemostat cultures were inoculated with *P. aeruginosa* strains PAO515 (*met-9011*, *amiE200*, *nalA5*) (66) and RM132 (76). Strain RM132 is lysogenic for the temperate bacteriophage F116 (48) and PAO515 serves as a recipient cell sensitive to infection by phage F116.

Media. Luria-Bertani (LB) agar plates were routinely used as a source of nonselective medium (45). Selective media consisted of LB agar containing nalidixic acid at 500 μg/ml. Batch and continuous culture microcosms contained *Pseudomonas* Minimal Medium lacking sodium citrate (PMM-c) with yeast extract added at 1×10^{-5}% (67).

Microcosms. Batch, continuous culture, and *in situ* incubated microcosms were established and maintained as described in Chapter IV.

Elimination of RM132 cells from microcosms by the addition of norfloxacin. In some experiments, the F116 lysogen was used as the initial source of F116 virion in the microcosm. In these cases, we used norfloxacin (Sigma Chemical Company, St. Louis, MO) to eliminate them from the microcosm. Submicrocosms were removed weekly from a batch culture

inoculated with RM132 at 1 x 10^6 CFU/ml and PAO515 at 1 x 10^7 CFU/ml. Norfloxacin was added at 500 µg/ml to each submicrocosm to terminate RM132 viability. Saye *et al.* (75) demonstrated that this treatment was effective in eliminating any additional virus production in norfloxacin-sensitive cells. Free phages were removed by centrifugation. The remaining culture of PAO515 (norfloxacin resistant) cells was spiked weekly and assayed for phage production and lysogenic properties.

Ultraviolet (UV) light studies. In some instances, samples were removed from batch cultures and exposed to UV light to determine whether starvation affected the activation of the lytic response in lysogens by DNA damage. Five milliliter samples were placed in sterile petri dishes (without covers) and exposed to 5 J/m^2 of UV-C radiation with a General Electric germicidal lamp (31). UV fluences were measured using a UVX radiometer (Ultraviolet Products, San Gabriel, CA). After exposure, samples were plated on LB agar and incubated overnight at 37°C in total darkness to prevent photoreactivation. Duplicate samples not exposed to UV light were also plated.

Other procedures. Phage and infective center enumerations, identification of phage-releasing and pseudolysogenic cells, and colony hybridizations were performed as explained in Chapter IV.

Results and Discussion

Effect of starvation on bacteriophage-host interactions. We added yeast extract to a starved culture containing *P. aeruginosa* strains PAO515 and RM132. After 55 days of starvation, a nutrient spike (yeast extract at a final concentration of 1 x 10^{-5}%) was added to the culture, simulating the feast-famine conditions typically encountered by microorganisms in the natural environment. Within one to two days of this spike, a small increase in the PBR and in the percentage of cells exhibiting lysogenic characteristics was seen (Fig. 11). Nutrient spikes continued on a weekly basis up to 110 days, with PBR and lysogen (measured by determining the number of clones releasing phage virions) increases occurring after each nutrient spike. No increases were observed in sister cultures that were not spiked with nutrient. These data illustrate that nutrient availability dramatically affects phage-host interactions, and, under starvation conditions, the establishment of true lysogeny and the production of virions are minimal at best.

Initial evidence of a pseudolysogenic state. We next established a microcosm culture containing strain PAO515 in PMM-c. We removed three submicrocosm cultures from this main microcosm after a 40-day period of starvation (Fig. 12). To each submicrocosm were added phage F116 virions, which were allowed to infect PAO515 cells over a 6 hour period after which all free virions were removed by centrifugation (75). In the first submicrocosm, yeast extract was added 24 hours after phage addition. In the second, yeast extract was added simultaneous to phage addition. And in the third, no nutrient

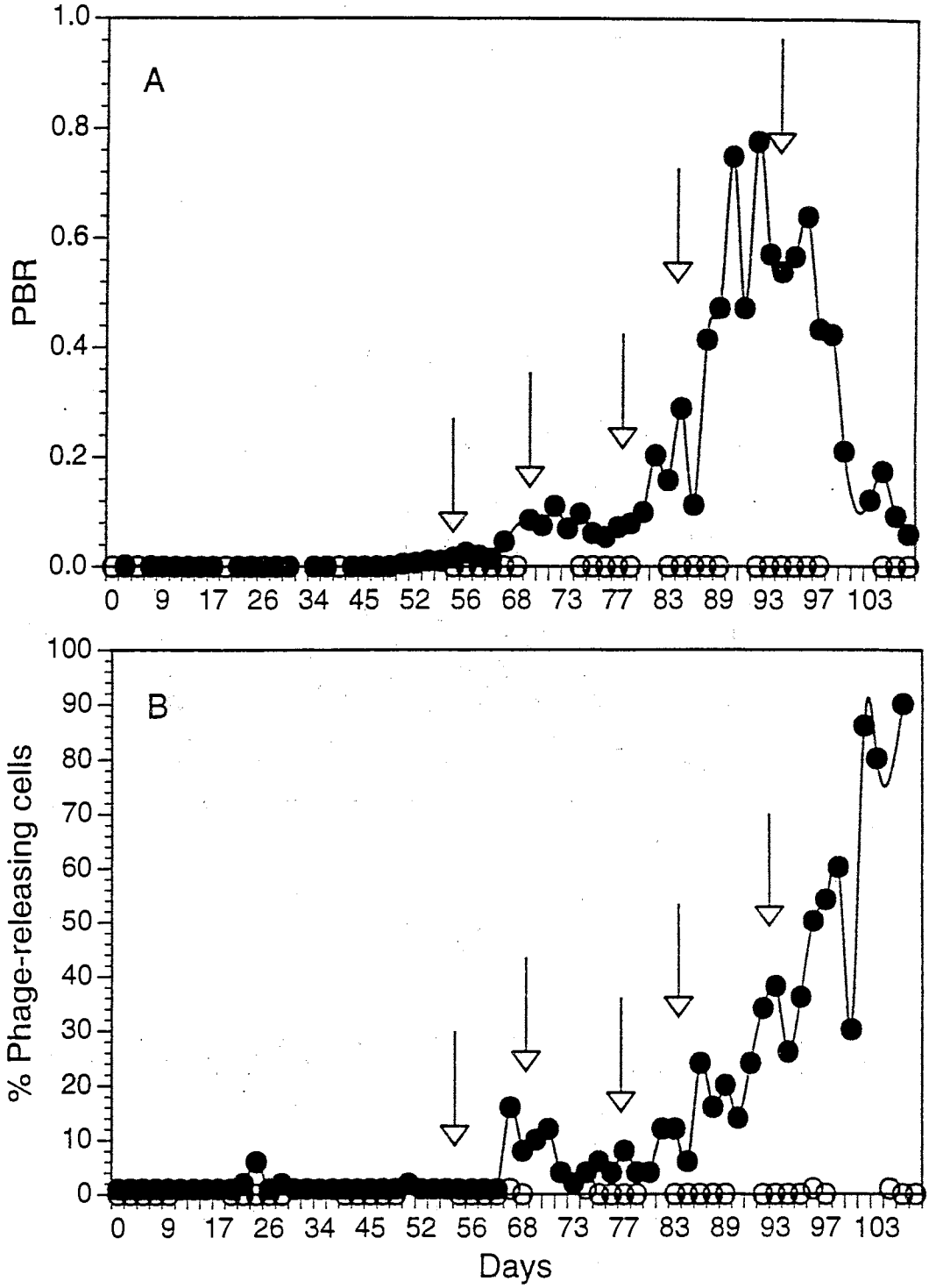


Fig. 11. Phage-to-bacterium ratio (PBR) and (B) percentage of cells actively releasing phage in a microcosm containing PMM-c and yeast extract at 1 x 10^{-5}%. Microcosms were inoculated with PAO515 and spiked with nutrient at times indicated by arrows. (O) No nutrient added; (●) Nutrient added.

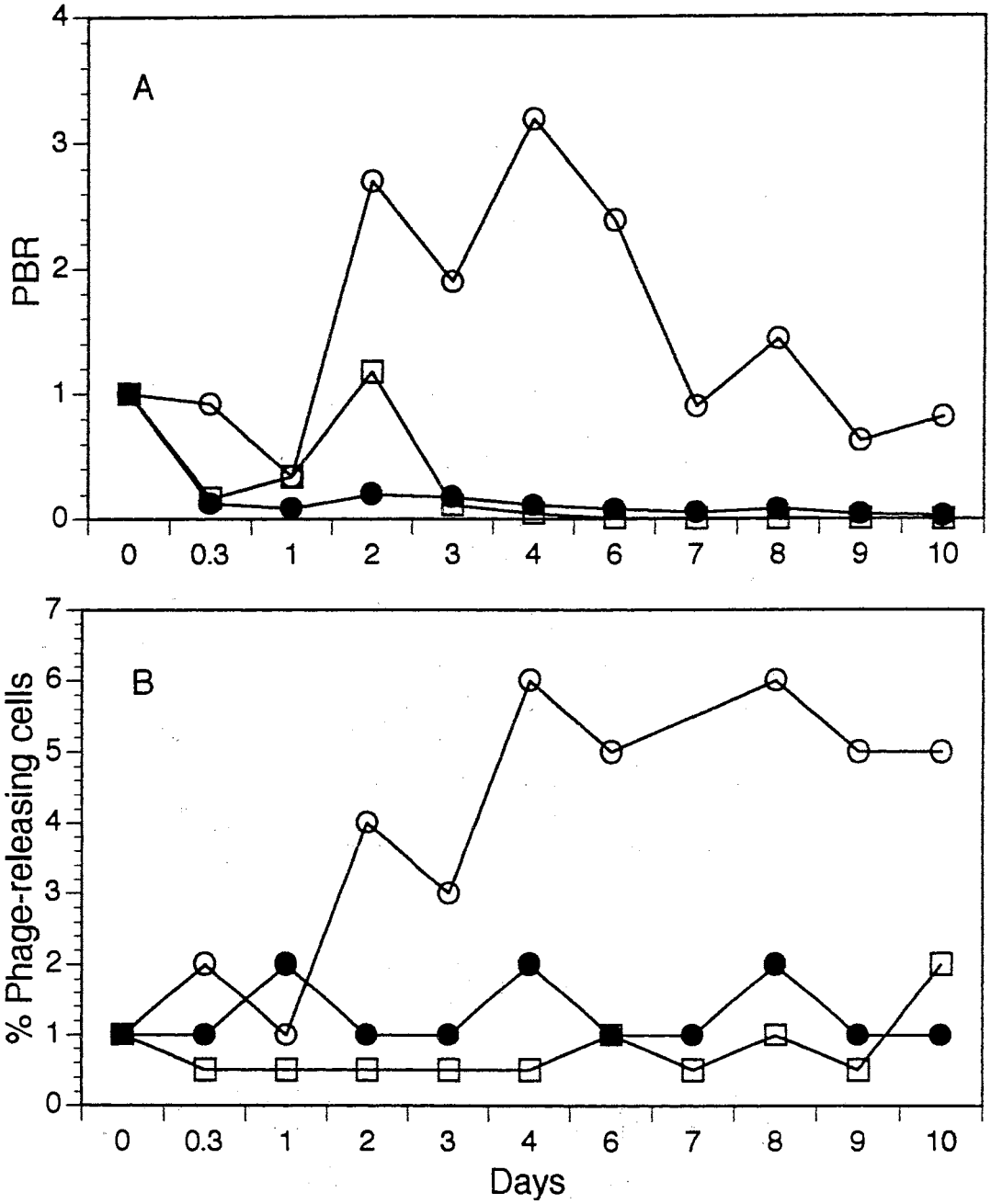


Fig. 12. Normalized (A) Phage-to-bacterium ratios (PBR) and (B) percentage of cells actively releasing phage in submicrocosms removed from a main batch culture inoculated with PAO515. (O) Bacteriophage F116 was added 24 h after submicrocosm was removed, allowed to infect for 6 h, and then removed by centrifugation. Yeast extract at a final concentration of 1 x 10^{-5}% was added 24 h later. (●) Bacteriophage F116 was added and removed as explained above. No yeast extract addition occurred. (□) Bacteriophage F116 was added and removed 6 h later. Nutrient was added simultaneous to phage addition.

was added. Our hypothesis of pseudolysogeny is that a phage infects a starved cell, but, due to the cell's starved state, there is not enough energy available for the phage to replicate itself. Therefore, enhanced virion production and increased frequency of phage-releasing clones should only be seen in the first submicrocosm where the phages were allowed to infect and subsequently reside within the cell in a non-active state. Once this occurs, the addition of nutrient 24 hours later will supply the required energy for the pseudoprophages to activate themselves to either the establishment of true lysogeny or lytic growth. Figure 12 shows that this does indeed occur. Simultaneous phage and nutrient addition had little effect on phage production and lysogeny establishment except for an expected small initial burst of phages. After this occurs, nutrient supplies are again exhausted and no further phage production develops. However, when phage virions were allowed to interact with the host before nutrient was added, a larger number of phages were produced and the frequency of true lysogeny was greatly increased over the results obtained with other treatments.

To further verify our theory, we performed another experiment in which strains PAO515 and RM132 were combined in a batch culture of PMM-c and allowed to starve over a 27 day period. Subcultures were removed on days 6, 12, 20, and 27. They were centrifuged to remove free phage particles and norfloxacin was added to eliminate viable RM132 cells and thus prevent further phage production in the culture (75). The remaining PAO515 cells were spiked with yeast extract at various intervals and assayed for their degree of phage production and frequency of lysogenized cells capable of releasing phage

(Table 8). The submicrocosms removed earliest, and thus containing the least-starved cells, displayed immediate phage production and lysogeny establishment. Although subcultures removed at later times exhibited increased lag times and required successively more nutrient spikes before phage and lysogen numbers increased, increases in these parameters occurred even in the 27-day culture (a total incubation time of 43 days). These data demonstrate that phage genomes are capable of a prolonged existence in a state of limbo within their starved hosts. We refer to such phage genomes as preprophages. As cellular starvation levels increase, so does the quantity of energy required to revitalize the cell and, in turn, activate the preprophage. From Table 8, we can see that as the days of incubation advance and the degree of starvation increases, successively larger amounts of energy are necessary for phage production and lysogen establishment.

Continuous culture. Continuous culture chemostats were used to maintain cultures of PAO515 and RM132 under idealized starvation conditions for extended periods of time (67). Again, PMM-c was used as the culture medium with yeast extract added at a final concentration of $1 \times 10^{-5}\%$ as a low-level carbon and energy source. Chemostats were run at a generation time (turn-over time) of 14 hours. Samples were removed and assayed for the number of cells actively releasing phage (i.e., true lysogens) and, through colony hybridization, the total number of cells containing phage F116 DNA. The difference between these two values represents the number of cells that contain F116 DNA but do not actively release phage (pseudolysogens). As

Table 8. Viable counts (CFU/ml), Phage-to-bacterium ratios (PBR) and percentage of cells releasing phage virions in submicrocosms removed from a main microcosm after 6, 12, 20, or 27 days. Submicrocosms were filtered to remove virions and norfloxacin was added to terminate production of new virions by induction of RM132 cells. Nutrient (1 x 10^{-5}% yeast extract, final concentration) was added at times indicated by an "X". Dashes (–) indicate that no sample was taken.

Day	Nutrient Added	Day 6			Day 12			Day 20			Day 27		
		CFU/ml	PBR ($x10^{-4}$)	% cells releasing phage	CFU/ml	PBR ($x10^{-4}$)	% cells releasing phage	CFU/ml	PBR ($x10^{-4}$)	% cells releasing phage	CFU/ml	PBR ($x10^{-4}$)	% cells releasing phage
0		$5.7x10^6$	0.09	<1	$1.4x10^7$	0.09	<1	$3.1x10^7$	0.01	<1	$4.4x10^7$	0.009	<1
1	X	$1.5x10^6$	2	<1	$8.8x10^6$	0.17	<1	$1.7x10^7$	0.01	<1	$1.9x10^7$	0.005	<1
2		$5.0x10^4$	54	<1	$1.3x10^6$	2	<1	$2.3x10^6$	15	<1	$1.4x10^7$	0.007	<1
4		$2.9x10^4$	107	<1	$5.9x10^5$	16	<1	$2.6x10^6$	3	<1	$1.1x10^7$	0.10	<1
6	X	$9.7x10^4$	16	<1	$1.6x10^5$	27	<1	$2.8x10^6$	1.0	<1	$1.0x10^7$	0.15	<1
8		$8.3x10^4$	87	<1	$1.7x10^4$	176	<1	$3.4x10^6$	1.6	<1	$1.0x10^6$	3	<1
10		$2.1x10^5$	62	11	$1.0x10^3$	1500	<1	$2.0x10^4$	650	<1	$6.7x10^5$	6	<1
12	X	$1.0x10^6$	61	72	$1.5x10^4$	180	<1	$2.0x10^2$	3150	<1	$6.7x10^5$	3	<1
14		$1.0x10^6$	8	65	$1.0x10^5$	3	<1	$5.0x10^4$	64	90	$1.3x10^4$	85	4
16		$1.6x10^7$	22	81	$1.0x10^6$	1	97	$8.4x10^6$	0.48	96	$6.7x10^3$	537	30
18		$1.4x10^7$	71	98	$1.6x10^7$	0.07	93	-	-	-	-	-	-
20		$2.3x10^7$	10	90	-	-	-	$1.1x10^7$	0.06	96	-	-	-

can be seen in Table 9, a large proportion (18 to 83%) of the population of cells which contained F116 DNA appeared to exist in a pseudolysogenic state. This is probably an under-estimation of the true proportion of pseudolysogenic cells in the starved chemostat because many of the cells releasing phage were most likely activated due to adsorption of nutrients during incubation on the plating medium.

Those colonies exhibiting pseudolysogeny were streaked onto Luria-Bertani agar plates to produce individual clonal colonies which were then colony hybridized with F116 DNA. In so doing, we discovered that only a small number of cells in the original pseudolysogenic colonies actually contained phage F116 DNA. Thus, it appears that the preprophage is not a stable entity within the pseudolysogenized cell. Presumably, due to the cell's starved state, there is not enough energy available for the infecting phage genome to become stably established in the host cell. Therefore, this preprophage is not replicated in synchrony with the host genome, similar to the process of abortive transduction, where the transduced allele does not integrate within it's host cell's genome (2). Only one of the two daughter cells from each division cycle acquires the exogenote allele.

***In situ* incubated microcosms.** *In situ* incubations were performed at Lake Sanborn, a small, semi-oligotrophic freshwater lake near Stillwater, Oklahoma, with a mean depth of approximately 2 m (18). Microcosms consisted of one-liter Lifecell tissue culture chambers (Fenwal Laboratories, Deerfield, IL) which were filled with 500 ml filter-sterilized (0.2 um) lake water and

Table 9: The frequency of pseudolysogens and true lysogens occurring in an ideally starved chemostat culture. See Text for details.

Day	Fraction of cells containing phage genome	% activated*	% not activated*
0	0/266	0	0
7	23/2000	100	0
10	51/850	17	83
15	403/1200	79	21
23	189/450	81	19
28	88/130	82	18
34	112/215	81	19

*Percent of cells which contain the phage genome that are either activated or not activated to release phage

inoculated with PAO515 and RM132 (76). Incubation occurred on the lake surface or at a 2 m depth. Some microcosms periodically received nutrient spikes of yeast extract at a final concentration of 1 x 10^{-5}%.

Only the microcosm incubated on the lake's surface and spiked with yeast extract exhibited large concentrations of cells infected with phage (Table 10). Of these, a high percentage contained phage DNA but were not actively releasing virions. The other microcosm on the surface which was not nutrient spiked produced few phage containing cells and very few pseudolysogenic cells. As was seen in the laboratory experiments, nutrient addition plays a vital role in phage-host interactions.

The microcosms incubated on the lake bottom produced virtually no phage-releasing cells. However, approximately one-to-two percent of the population routinely exhibited pseudolysogenic characteristics. In the nutrient-spiked microcosm, this value had risen to 10% by the end of the experiment when cells were most starved. The lack of phage-producing cells in these microcosms was most likely due to the lack of exposure to ultraviolet (UV) radiation, which has previously been shown to induce F116 prophages to lytic growth (26, 31). Regan *et al.* (65) have shown that the biologically effective DNA-damaging dose of UVB (activating solar UV wavelengths) only penetrates to 2 m or less in estuarine waters. Our microcosms incubated on the lake bottom resided at a depth of 2 m in very cloudy water and were unlikely to be affected by solar UV light.

To test whether starvation eliminates the ability of UV to induce F116 prophage from true lysogens, we exposed phage F116-infected cells to 5 J/m^2

Table 10. *In situ* microcosms incubated in Lake Sanborn near Stillwater, Oklahoma either on the lake surface or at a depth of 2 m. Yeast extract was periodically added to one of each of the surface and bottom microcosms.

Day	Surface						Bottom					
	No Nutrient Added			Nutrient Added			No Nutrient Added			Nutrient Added		
	Fraction of cells containing phage genome	% activated*	% not activated*	Fraction of cells containing phage genome	% activated	% not activated	Fraction of cells containing phage genome	% activated	% not activated	Fraction of cells containing phage genome	% activated	% not activated
0	0/840	0	0	0/580	0	0	4/660	0	100	0/910	0	0
6	6/630	100	0	9/660	0	100	9/900	0	100	5/440	0	100
7	20/1600	100	0	3/520	0	100	1/93	0	100	7/500	0	100
9	9/1200	100	0	1/180	0	100	22/1100	0	100	1/140	0	100
12	3/530	100	0	7/350	0	100	6/870	0	100	11/2100	0	100
14	5/900	100	0	11/183	93	7	4/380	0	100	3/580	0	100
21	53/3000	50	50	323/449	86	14	8/795	0	100	14/1900	0	100
28	6/120	80	20	377/670	93	7	-	-	-	2/160	0	100
35	5/860	100	0	261/287	81	19	30/1200	0	100	8/240	0	100
41	1/110	100	0	365/406	78	22	2/210	0	100	81/800	20	80

*Percent of cells which contain the phage genome that are either activated or not activated to release phage

of UV-C radiation over a 36 day starvation period (Fig. 13). Prophages remained inducible throughout this time.

Conclusions

We have shown in this study that pseudolysogenic relationships between temperate bacteriophages and their hosts are possible and actually appear quite prevalent in environmental ecosystems where the effects of nutrient limitation augment the response. These results are consistent with earlier observations we have made on pseudolysogeny in environmental interactions between lytic bacteriophages and their hosts (70). As a direct result, bacteriophages are able to survive within their hosts for extended periods. This prolonged life span of phage genomes in the environment may provide an expanded natural reservoir of viruses leading to the large phage populations reported by other investigators (6, 9, 20, 63, 64). Our laboratory has previously shown that transduction, the transfer of genetic material by bacteriophages, is routinely encountered in natural ecosystems and therefore must be taken into consideration when assessing the risks involved with gene transfer between genetically engineered microorganisms and the indigenous microbial population (46, 49). Pseudolysogeny may increase potential risk by increasing the probability of exposure of the natural population to transduction events, thereby increasing the potential for horizontal gene transfer between introduced and natural populations of bacteria.

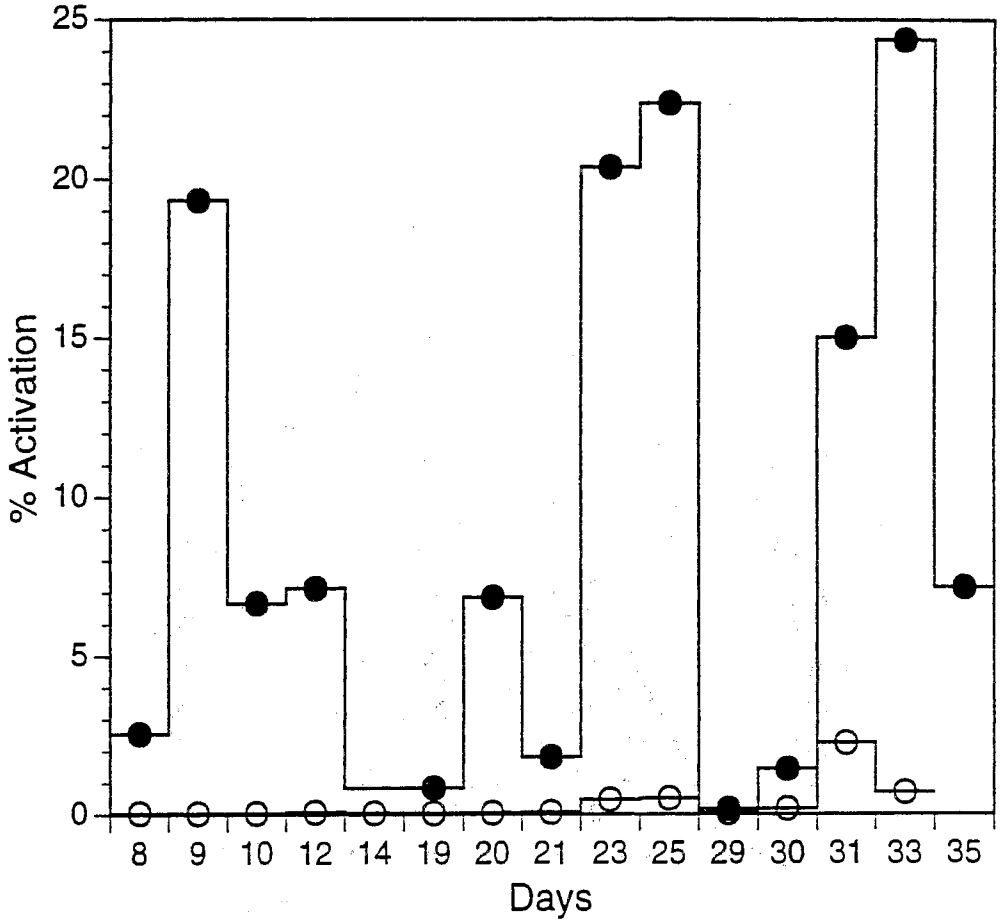


Fig. 13. Induction of starved F116-infected cells by ultraviolet (UV) light. (O) Cells not exposed; (●) Cells exposed to 5 J/m^2 UV-C.

CHAPTER VI

DYNAMICS OF THE PSEUDOLYSOGENIC RESPONSE IN SLOWLY GROWING CELLS

Introduction

Our laboratory has been attempting to construct a model of bacteriophage-host interactions in natural environments. Previous models have relied on data gathered from experiments in which well-fed cells were analyzed under laboratory growth conditions. Our studies, using low-nutrient parameters and generation times typically encountered in natural ecosystems, have revealed a remarkably different type of phage-host interaction referred to as pseudolysogeny (3). Pseudolysogeny describes a condition in which the starved host cell coexists with its viral genome for extended periods. As nutrient supplies are replenished, the pseudolysogenized cell induces true lysogeny or becomes activated to produce and release virions. The direct result of pseudolysogenic relationships is the extension of phage half-lives, and, ultimately, the development of a way in which phages can maintain themselves in nutrient-deprived environments in the large numbers documented by several recent studies (6, 9, 20, 63, 64, 87).

In the study reported here, we have utilized continuous-culture chemostats to quantitatively assess the pseudolysogenic response in cells growing under increasingly more severe states of starvation. These states were produced by lengthening the hydraulic turnover (generation) times of the chemostat culture. In this manner, we assessed pseudolysogenic interactions over a wide range of growth limitations to see how these responses correlate to the development of the pseudolysogenic response.

Materials and Methods

Bacterial strains and bacteriophages. *P. aeruginosa* strains PAO1 (prototrophic) (45), PAO303 (*argB21*) (29), PAO515 (*met-9011, amiE200, nalA5*) (66), and RM132 (an F116 lysogen of PAO303) (76) were used in this study. Bacteriophage F116 is a temperate, generalized transducing phage (48). Phage UT1 was isolated from a natural freshwater lake (59). It exhibits virulence under laboratory conditions, but appears pseudolysogenic under natural conditions (32). It is also a generalized transducing phage (71).

Media. Luria-Bertani (LB) agar plates were routinely used as nonselective medium (45). Selective media consisted of either LB agar containing nalidixic acid (500 μg/ml) or *Pseudomonas* Minimal Medium (PMM) containing 0.4% glucose supplemented with arginine at 25 μg/ml (45). Chemostats contained a variation of PMM in which the sodium citrate was omitted (PMM-c). Yeast extract was added at various concentrations, serving as the sole source of carbon and nitrogen in these continuous-culture experiments.

Continuous-culture conditions. New Brunswick Bioflo Model C30 bench top chemostats (New Brunswick Scientific Co. Inc., Edison, NJ) were utilized in these experiments. Chemostats consisted of a 1 l or 1.5 l culture vessel and a 10 l medium reservoir containing PMM-c supplemented with yeast extract at final concentrations ranging from 10^{-2}-to-10^{-6}% (final concentration). Generation times varied from 5-to-14 hours. Incubations were performed at room temperature at an agitation rate of 200 rpm.

Preparation of inoculants. Strains inoculated into chemostats were first grown overnight at 37°C in LB broth and then diluted 1/100 in PMM-c supplemented with 0.5% yeast extract and grown to mid-exponential phase at 37°C. Cultures were then washed twice in PMM-c (containing no yeast extract) and inoculated into the chemostats. For studies involving phage F116, strains PAO515 and RM132 were inoculated at approximately 1 x 10^6 and 1 x 10^5 CFU/ml, respectively, into the same culture vessel. Studies involving phage UT1 utilized strain PAO303 inoculated at approximately 1 x 10^6 CFU/ml. After a stabilization period of four days, phage UT1, suspended in PMM-c, was added at 1 x 10^5 PFU/ml.

Other procedures. Phage and infective center enumerations, identification of phage-releasing and pseudolysogenic cells, and colony hybridizations were performed as explained in Chapter IV.

Results

Several studies have assessed phage-host interactions in chemostat cultures (13, 33, 37, 38). However, none have examined the pseudolysogenic response under continuous-culture conditions. The nutrient concentrations and generation times established for the numerous chemostat cultures described here were our best approximations of what might occur in a natural freshwater lake (67). However, in a typical environmental situation, parameters affecting population growth characteristics are essentially too widely varied to undergo precise measurement. Therefore, an extended range of nutrient concentrations and generation times were used in the chemostat simulations in the hopes of achieving an overall view of phage-host interactions in the environment. Growth rates for each of the strains used in these experiments were established in filter-sterilized lake water (Fig. 14). Strains typically maintained their populations at levels comparable to those which developed in the chemostats. Exceptions occurred at the extreme low end of nutrient concentration range. In general, growth in the chemostats was maintained at approximately 10^5-to-10^8 CFU/ml and 10^3-to-10^9 PFU/ml, which closely approximates the concentration found in natural aquatic populations (6, 50, 64, 82).

Estimation of bacteriophage F116 activities at varying nutrient concentrations. Figure 15A-E presents bacterial and phage F116 growth patterns in chemostats supplemented with yeast extract at various concentrations. As is readily apparent, phage and bacterial populations are maintained at the highest densities at high yeast extract concentrations and decrease as

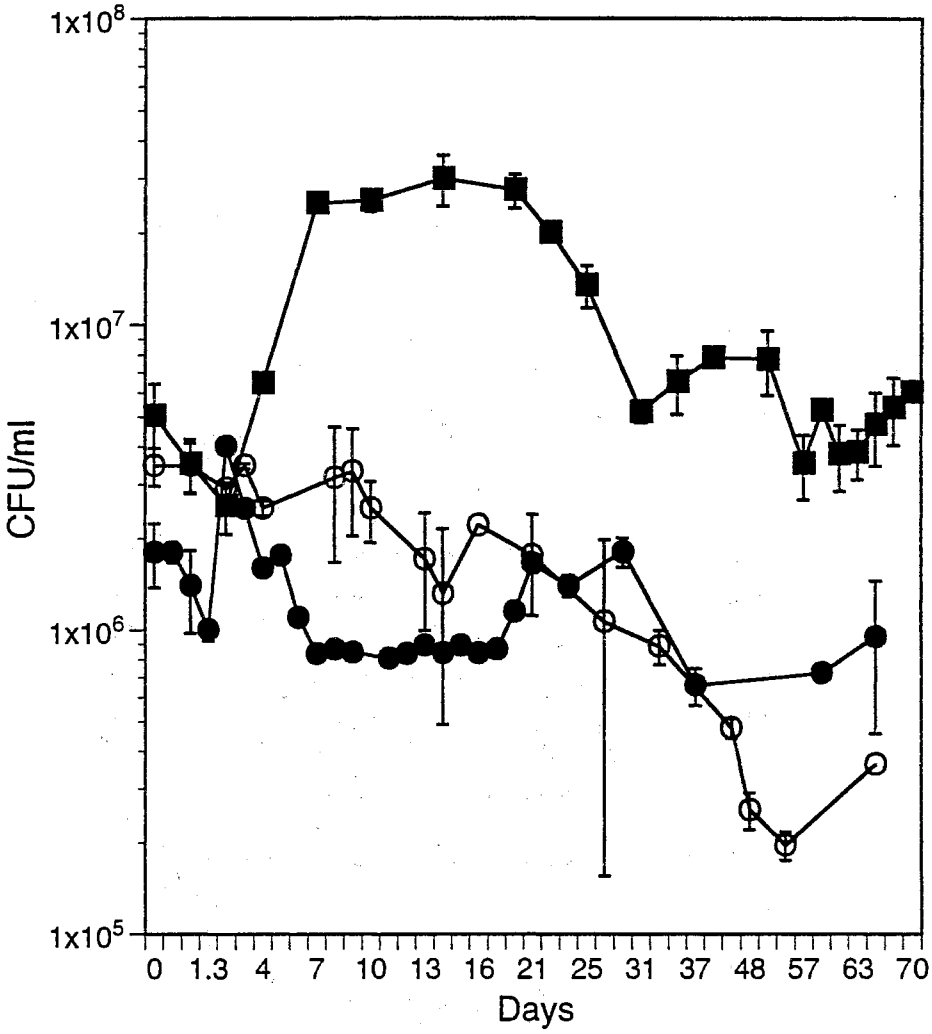


Fig. 14. Growth curves of *P. aeruginosa* strains *PAO515* (●), RM132 (O), and PAO303 (■) in lake water. Incubations occurred at room temperature with slow shaking for approximately 70 days.

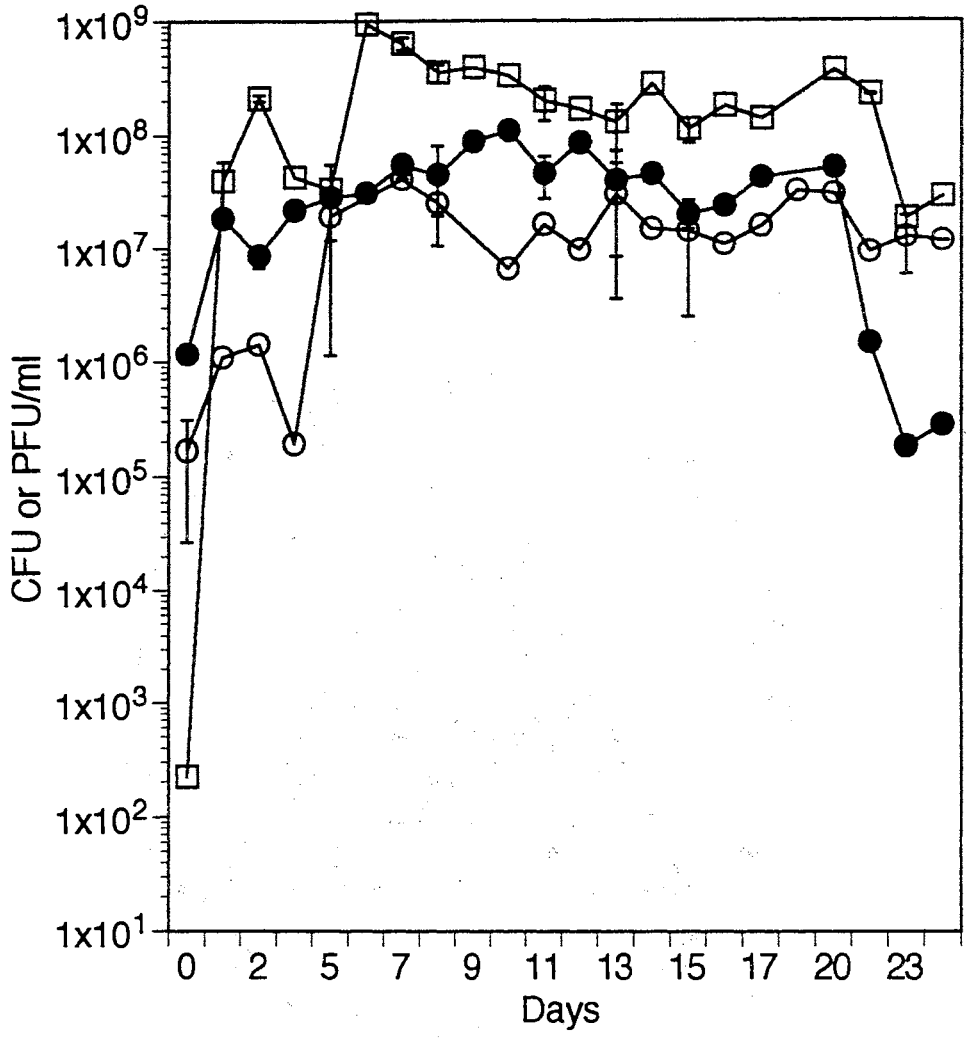


Fig. 15A. Chemostat microcosm containing yeast extract at a final concentration of 1 x 10^{-2}%. (O) RM132 (F116 lysogen), (●) PAO515, (□) Phage F116

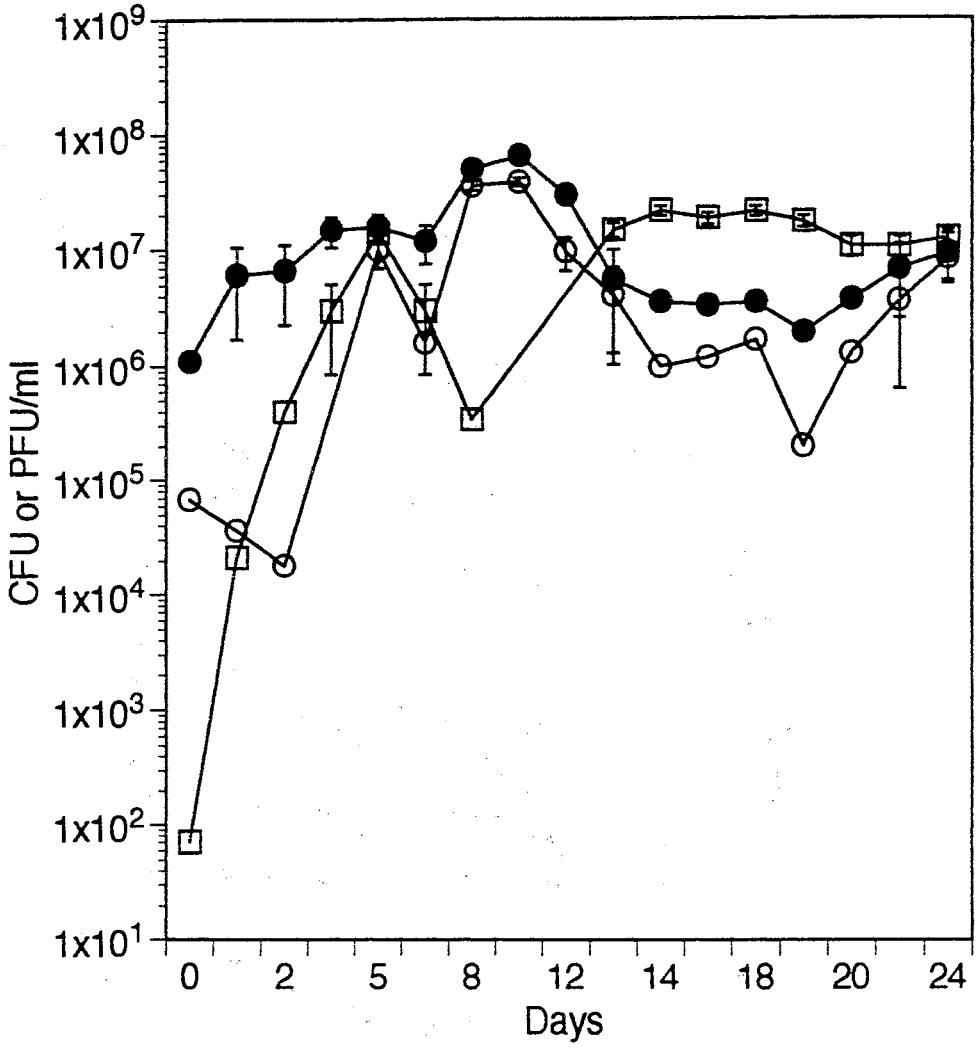


Fig. 15B. Chemostat microcosm containing yeast extract at a final concentration of 1 x 10^{-3}%. (○) RM132 (F116 lysogen), (●) PAO515, (□) Phage F116

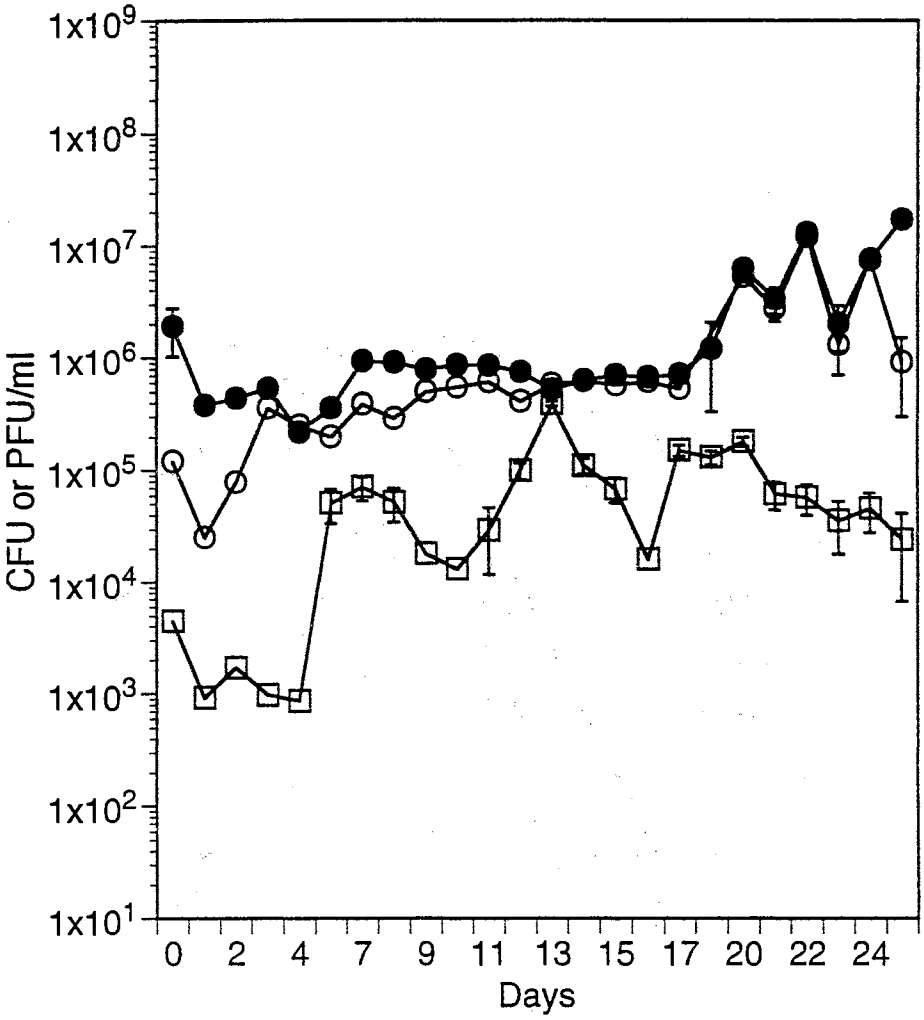


Fig. 15C. Chemostat microcosm containing yeast extract at a final concentration of 1 x 10^{-4}%. (O) RM132 (F116 lysogen), (●) PAO515, (□) Phage F116

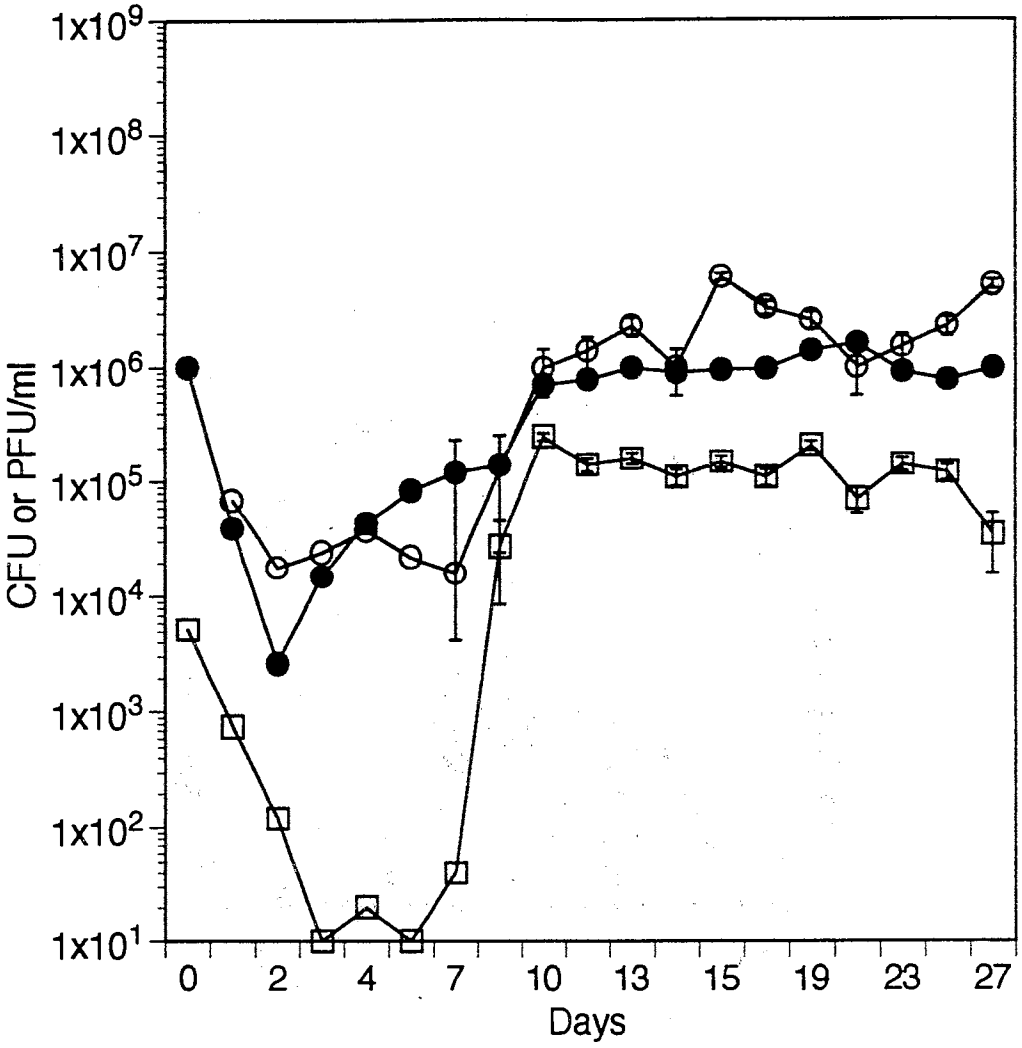


Fig. 15D. Chemostat microcosm containing yeast extract at a final concentration of 1 x 10^{-5}%. (O) RM132 (F116 lysogen), (●) PAO515, (□) Phage F116

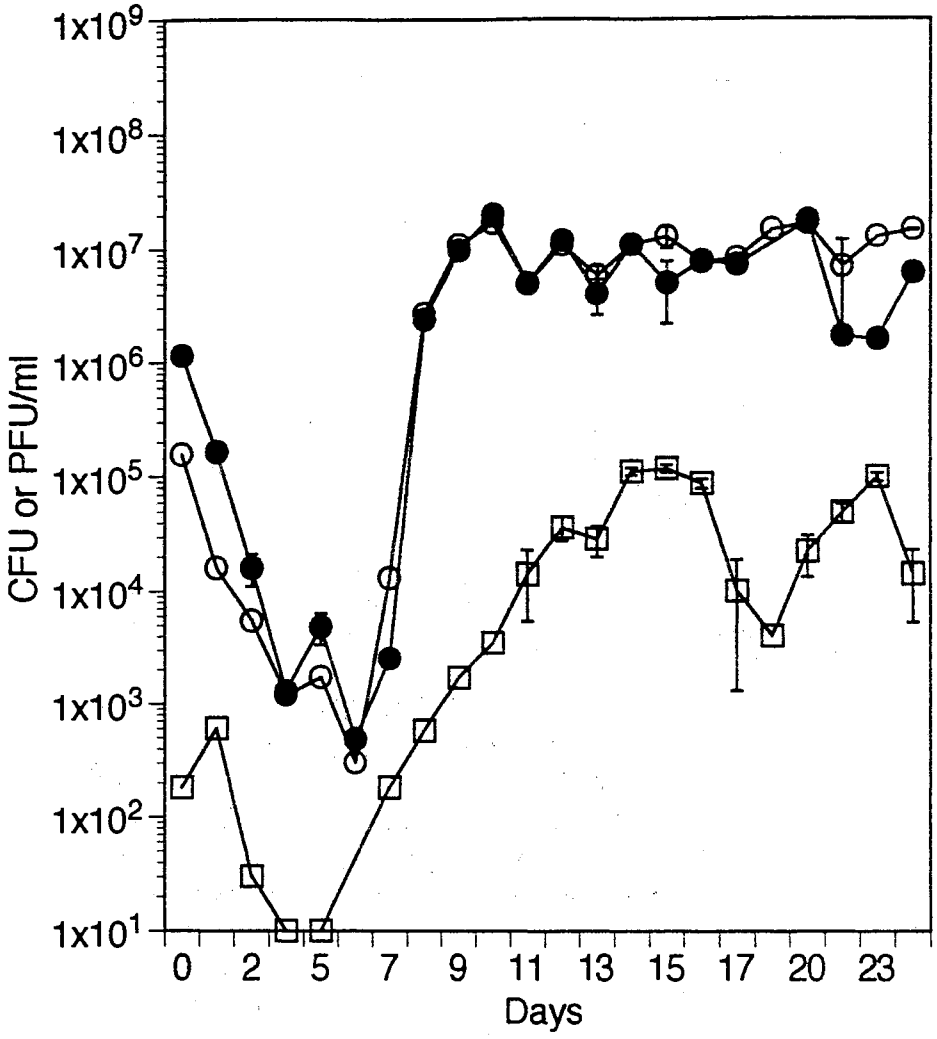


Fig. 15E. Chemostat microcosm containing yeast extract at a final concentration of 1×10^{-6}%. (O) RM132 (F116 lysogen), (●) PAO515, (□) Phage F116

the concentration of yeast extract decreases. Table 11 lists the PBRs that developed over the course of each chemostat run. A rather dramatic difference exists between phage production at high and low nutrient concentrations. However, the exact opposite observation is made when assessing pseudolysogen concentrations (Table 12). At 1 x 10^{-2}% yeast extract, the frequency of cells that have been pseudolysogenized is minimal but gradually increases until a maximum is attained at 1 x 10^{-6}% yeast extract.

According to our theory of pseudolysogeny, this is to be expected. As cellular starvation levels increase, we would predict that the occurrence of pseudolysogeny would also increase. It is interesting to note that the percentage of cells found to contain the F116 genome (those testing positive in colony hybridizations) increased as nutrient levels decreased. The degree of increase between 10^{-2}-and-10^{-3}% yeast extract did not, in general, substantialy differ. However, there was a difference between these concentrations and the rates observed at 10^{-5}-and-10^{-6}%. These observations correlate well with the larger numbers of pseudolysogens present in the 10^{-5}-and-10^{-6}% yeast extract experiments.

Estimation of bacteriophage UT1 activities at varying nutrient concentrations. When the virulent phage UT1 was grown at various nutrient concentrations, equilibriums were established between phage and host numbers in all cases (Fig. 16A-E). At 1 x 10^{-6}% yeast extract, equilibrium was maintained for only a brief period after which phage concentrations declined. However, as phage numbers declined, the number of cells containing phage

Days	Final yeast extract concentration				
	10^{-2}%	10^{-3}%	10^{-4}%	10^{-5}%	10^{-6}%
0	<1	<1	2	5	<1
1	1452	3	3	19	3
4	1593	188	3	<1	2
8	4667	7	47	<1	<1
10	2538	ND	11	<1	<1
12	2152	ND	143	179	4
14	4667	2885	180	122	8
16	5806	5758	195	113	11
19	ND	6000	108	150	<1
23	1462	1571	18	154	7
26	857	929	2	156	1

Table 11. Phage-to-bacteria ratios (PBRs) ($x10^{-3}$) in chemostats inoculated with RM132 and PAO515 at varying concentrations of yeast extract. ND, Not determined

Day	1 x 10^{-2}% yeast extract			1 x 10^{-3}% yeast extract			1 x 10^{-4}% yeast extract			1 x 10^{-5}% yeast extract			1 x 10^{-6}% yeast extract		
	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated
0	1/280	100	<1	1/330	100	<1	1/220	100	<1	1/290	100	0	2/140	50	50
9	90/300	96	4	2/300	100	<1	122/1140	82	18	103/400	50	50	60/250	57	43
13	46/340	96	4	40/430	98	2	62/380	89	11	146/580	75	25	166/420	67	33
17	52/400	90	10	52/620	94	6	58/290	45	55	186/460	63	37	110/260	56	44
25	82/280	93	7	38/470	95	5	92/360	83	17	92/230	61	39	127/250	59	41

Table 12. Comparison of the percent of phage-releasing cells (% activated) to pseudolysogenic cells (% not activated) in chemostat microcosms inoculated with Rm132 and PAO515 at varying concentrations of yeast extract.

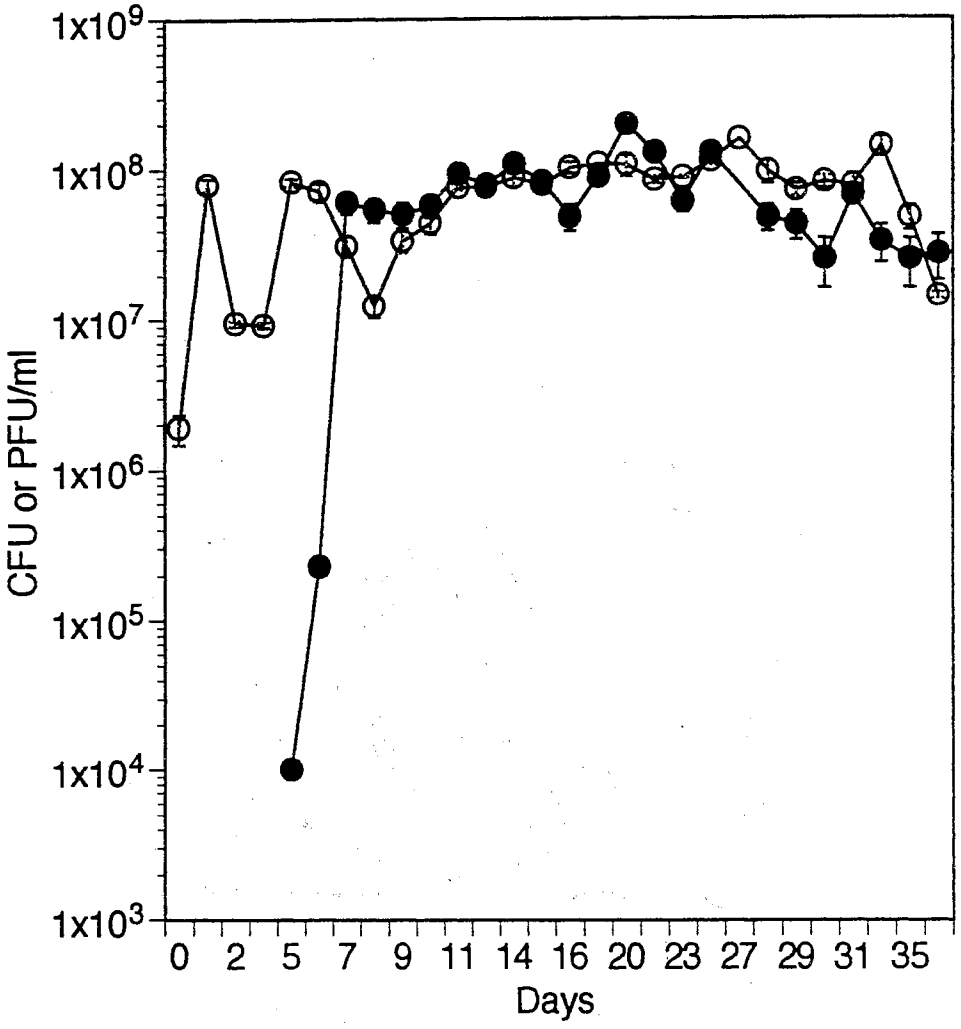


Fig. 16A. Chemostat microcosm containing yeast extract at a final concentration of 1 x 10^{-2}%. (O) PAO303, (●) Phage UT1

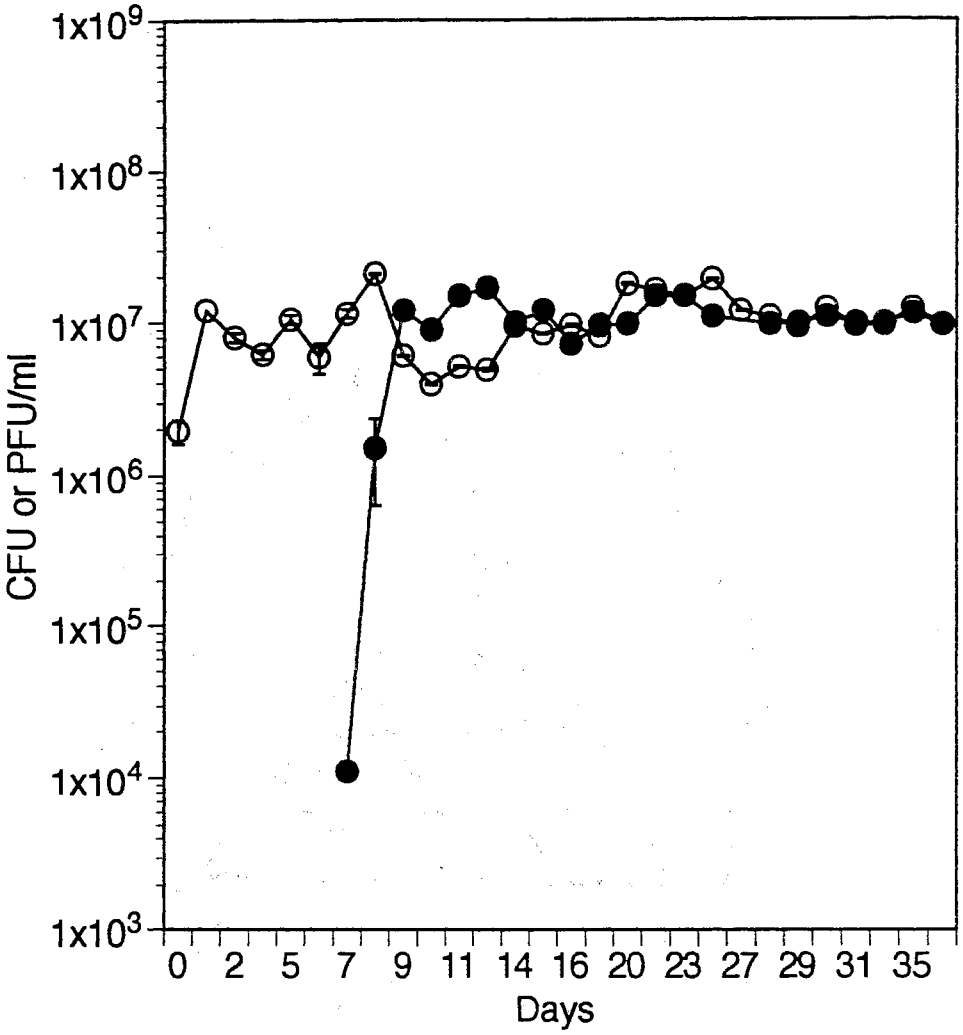


Fig. 16B. Chemostat microcosm containing yeast extract at a final concentration of 1 x 10^{-3}%. (O) PAO303, (●) Phage UT1

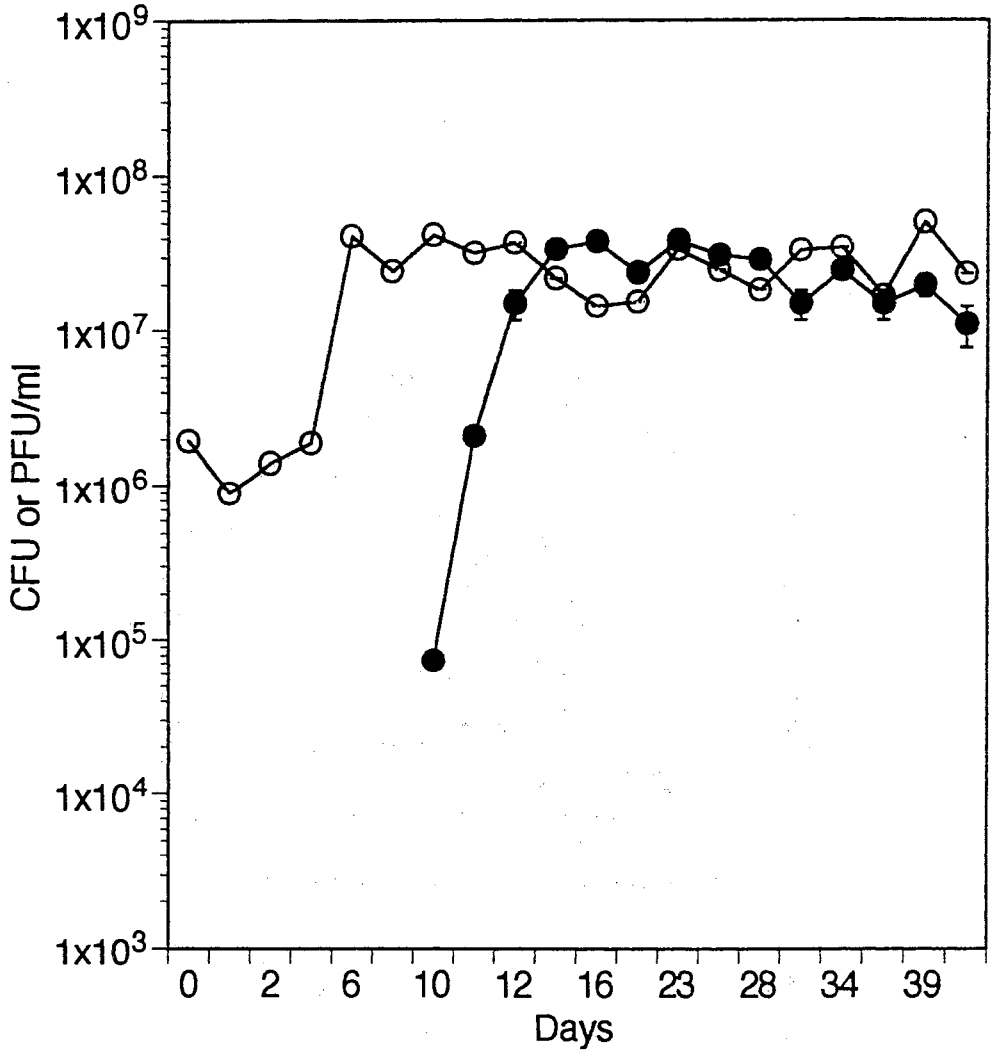


Fig. 16C. Chemostat microcosm containing yeast extract at a final concentration of 1 x 10^{-4}%. (O) PAO303, (●) Phage UT1

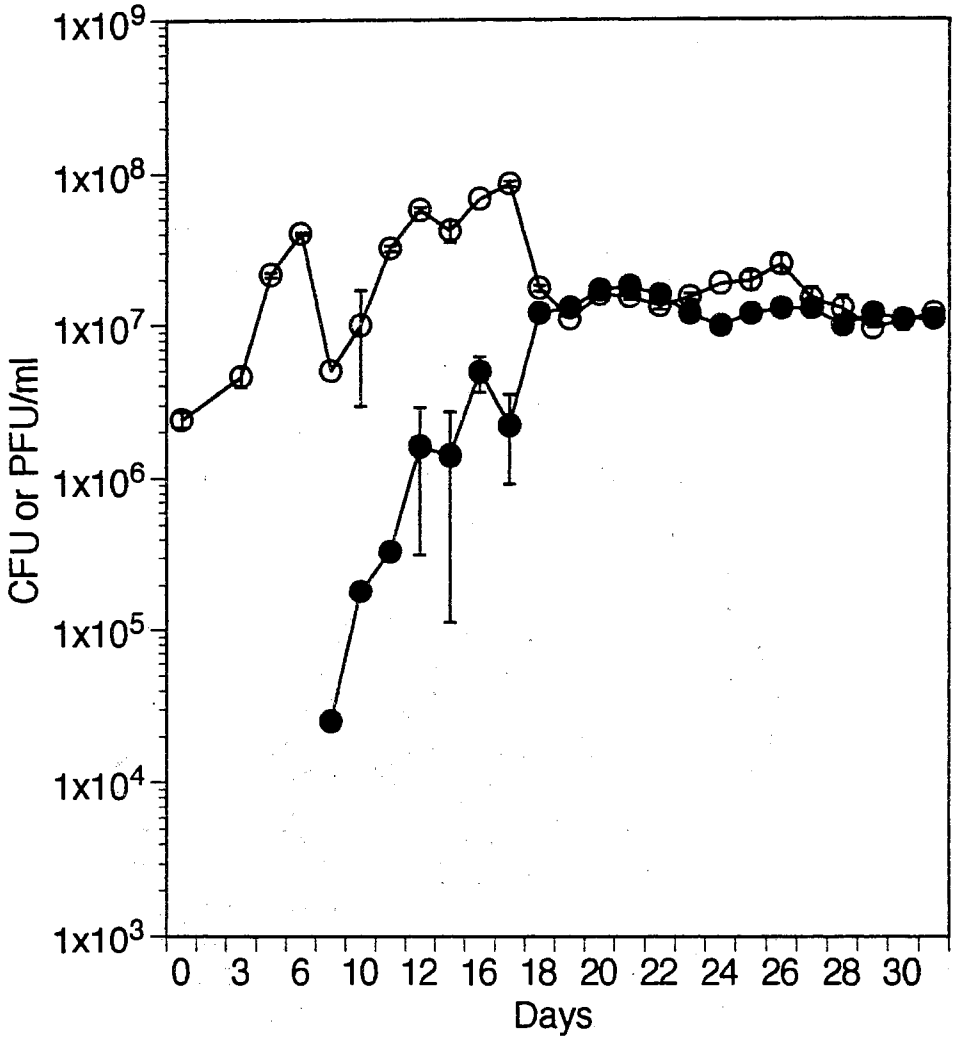


Fig. 16D. Chemostat microcosm containing yeast extract at a final concentration of 1 x 10^{-5}%. (O) PAO303, (●) Phage UT1

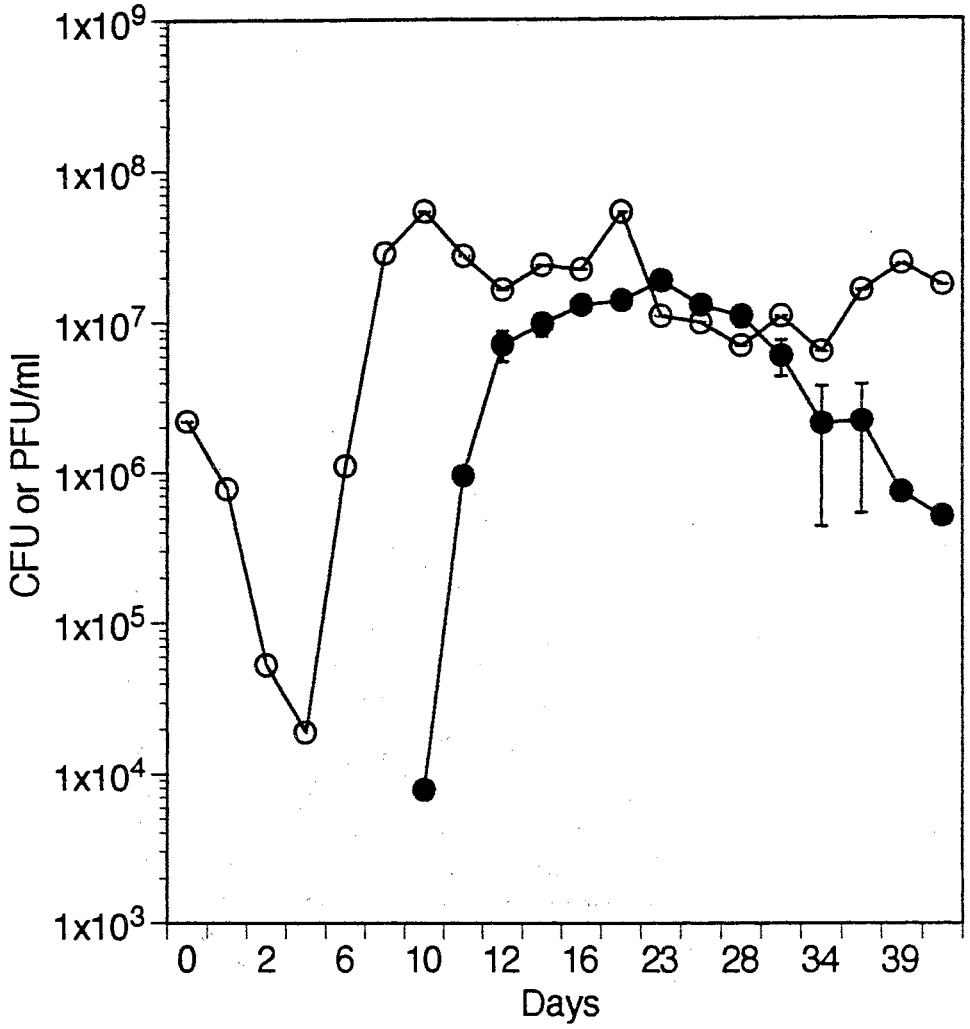


Fig. 16E. Chemostat microcosm containing yeast extract at a final concentration of 1 x 10^{-6}%. (O) PAO303, (●) Phage UT1

DNA increased, of which approximately 30-to-50% were pseudolysogenic (Table 13). Pseudolysogenic concentrations this large were seen in none of the other microcosms, emphasizing the importance of nutrient limitation to pseudolysogen establishment. In fact, the overall percentage of cells exhibiting pseudolysogenic characteristics increased rather dramatically from high to low yeast extract additions, just as was seen for phage F116. PBRs generally followed the same pattern as for F116, as well (Table 14). At high nutrient levels, high PBR concentrations were attained almost immediately. As nutrient levels decreased, PBR establishment was delayed.

Estimation of bacteriophage F116 activities at varying generation times. The generation time for a continuous culture is defined as the time it takes for the culture vessel to completely replenish itself with medium (41). Mathematically, this is represented as the reciprocal of the dilution rate, 1/D, or the ratio of the volume of the culture to the rate at which fresh medium flows into the culture (V/F). Therefore, at short generation times (i.e. 5 hours), cells are provided with fresh medium at a rapid rate and growth proceeds under conditions of only mild nutrient limitation. As generation times increase, the flow rate of fresh medium decreases such that nutrient addition occurs at a very slow rate. Consequently, the growth environment is severely nutrient limited and cells exist in a starved state. For the experiments reported here, generation times of 5, 8, 10, 12, and 14 hours were used at a constant yeast extract concentration of 1×10^{-4}% (Fig. 17A-E).

Day	1 x 10^{-2}% yeast extract			1 x 10^{-3}% yeast extract			1 x 10^{-4}% yeast extract			1 x 10^{-5}% yeast extract			1 x 10^{-6}% yeast extract		
	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated
10	83/400	91	9	125/420	95	5	54/460	90	10	30/280	78	22	33/360	92	8
15	92/770	92	8	137/880	92	8	282/830	85	15	75 /1100	80	20	412 /2600	88	12
22	20/820	100	<1	360 /1800	98	2	62/280	84	16	78 /1300	76	24	342 /1100	72	28
28	60/860	100	<1	294 /1200	95	5	42/400	84	16	45/190	80	20	330 /660	66	34
35	87/430	100	<1	52/120	94	6	100/230	85	15	82/130	75	25	40/100	54	46

Table 13. Comparison of the percent of phage-releasing cells (% activated) to pseudolysogenic cells (% not activated) in chemostat microcosms inoculated with PAO303 and bacteriophage UT1 at varying concentrations of yeast extract.

Days	Final yeast extract concentration				
	10^{-2}%	10^{-3}%	10^{-4}%	10^{-5}%	10^{-6}%
0	0	0	0	0	0
7	194	<1	ND	<1	ND
9	150	198	ND	ND	ND
10	129	228	<1	<1	<1
13	104	347	155	3	41
16	47	75	262	7	58
17	82	118	ND	4	17
20	185	54	155	106	26
23	70	100	115	75	173
29	59	106	157	124	156
30	31	88	ND	113	ND
31	87	105	45	92	55
34	23	104	71	ND	33
35	53	93	88	ND	13

Table 14. Phage-to-bacteria ratios (PBRs) (x10^{-2}) in chemostats inoculated with PAO303 and bacteriophage UT1 at varying concentrations of yeast extract. ND, Not determined

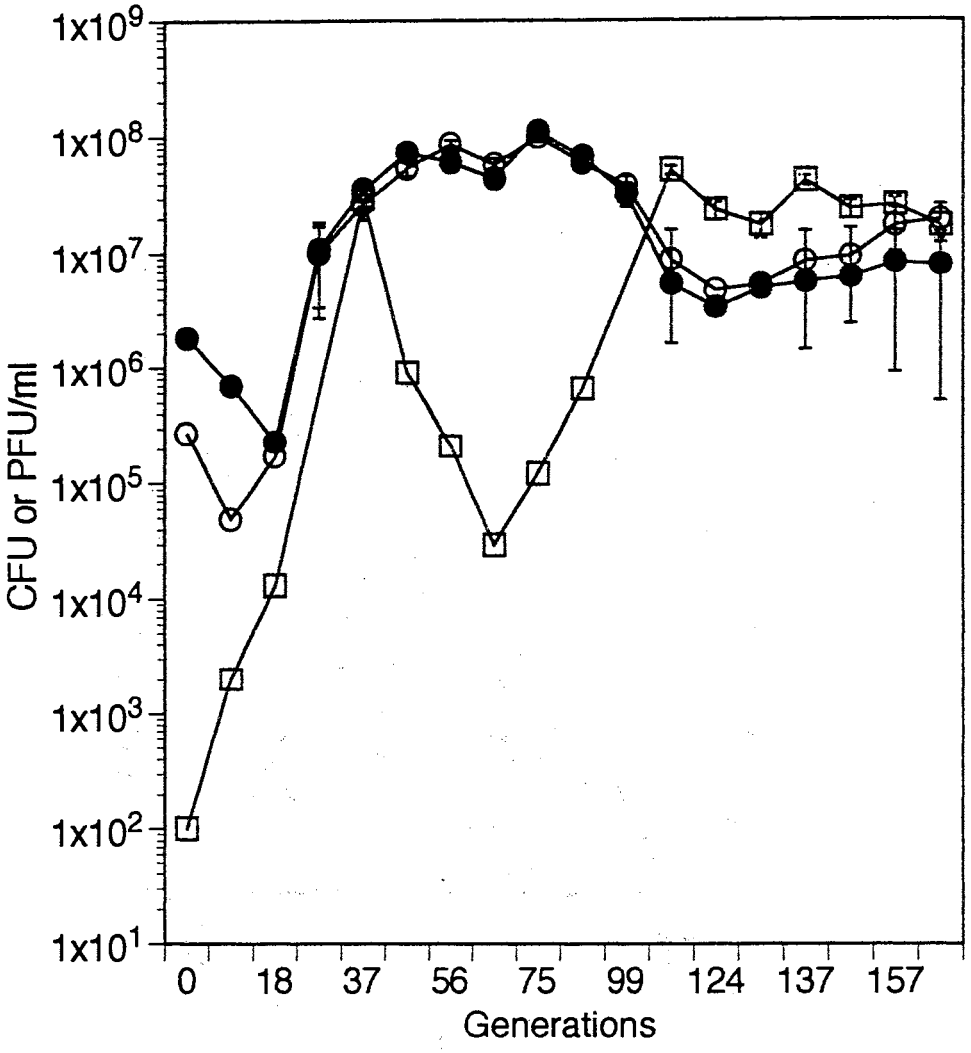


Fig. 17A. Chemostat microcosm incubated at a generation time of 5 hours. (O) RM132 (F116 lysogen), (●) PAO515, (□) Phage F116

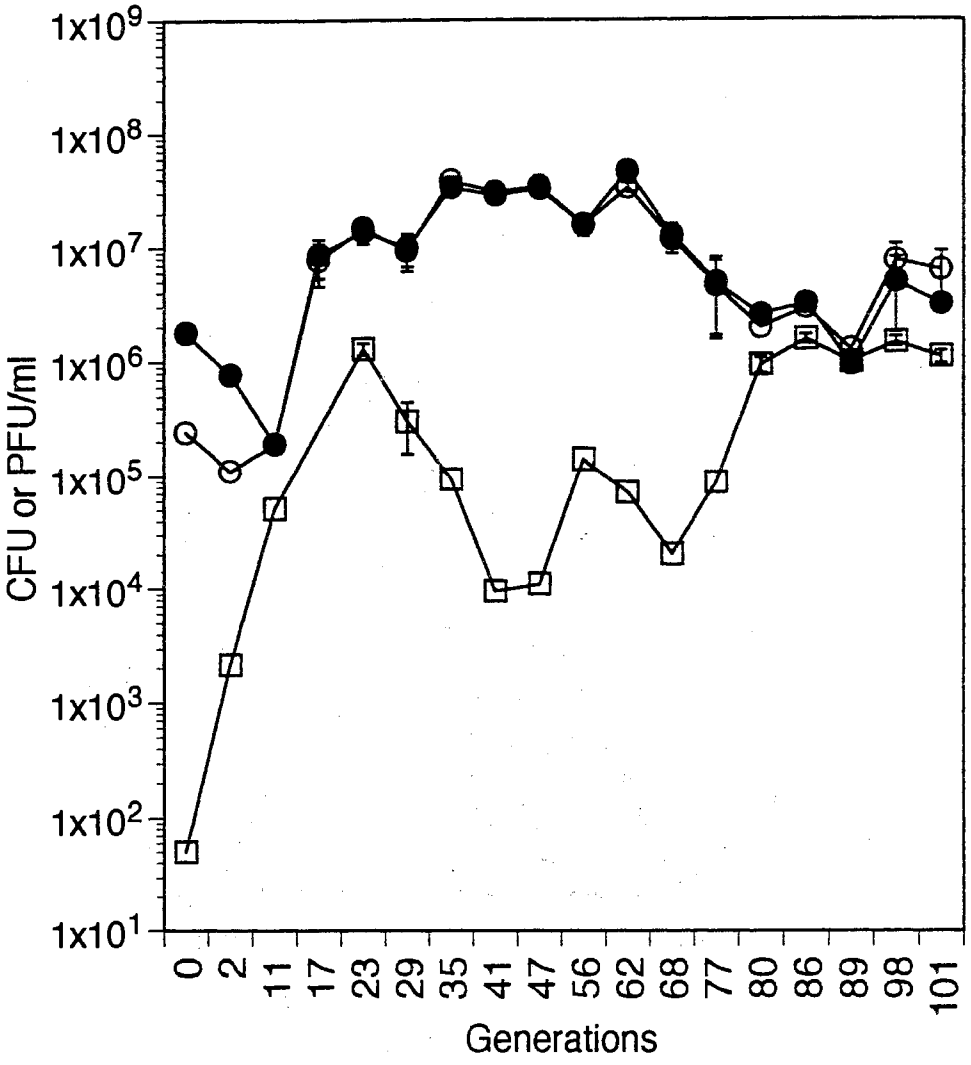


Fig. 17B. Chemostat microcosm incubated at a generation time of 8 hours. (○) RM132 (F116 lysogen), (●) PAO515, (□) Phage F116

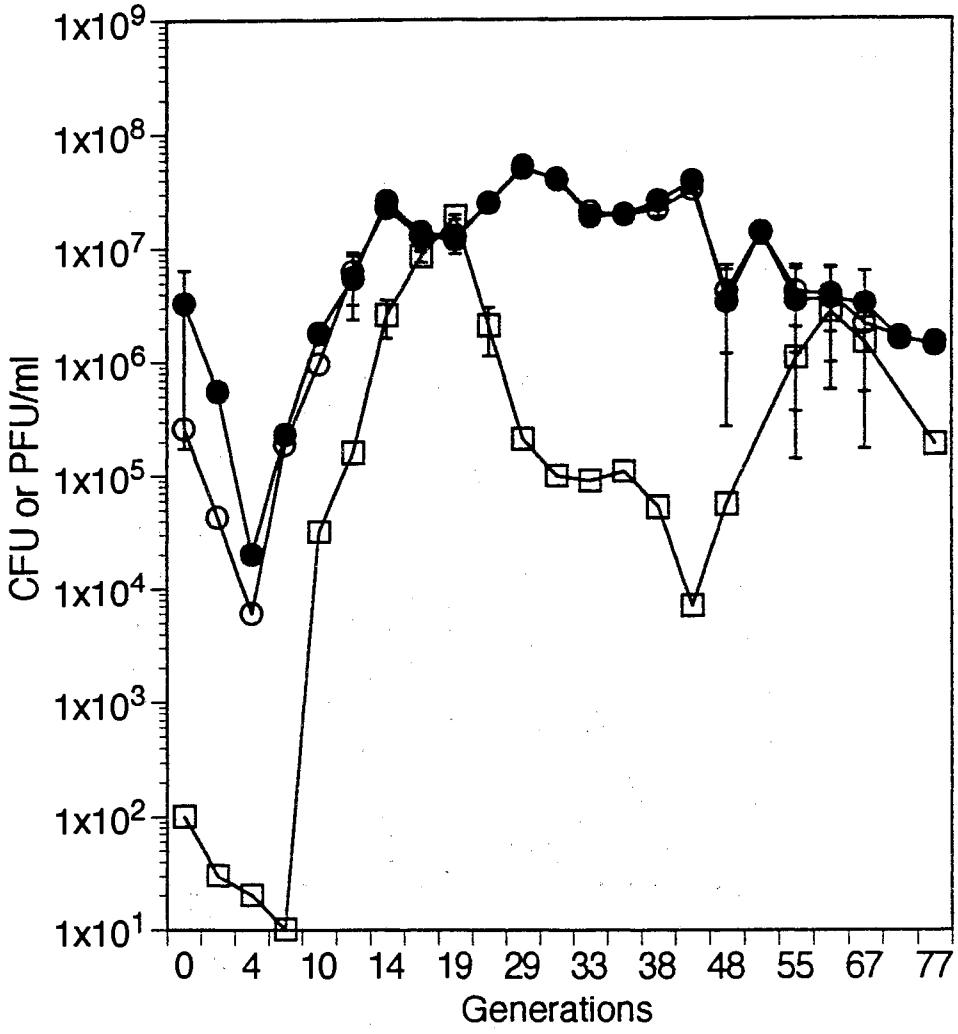


Fig. 17C. Chemostat microcosm incubated at a generation time of 10 hours. (O) RM132 (F116 lysogen), (●) PAO515, (□) Phage F116

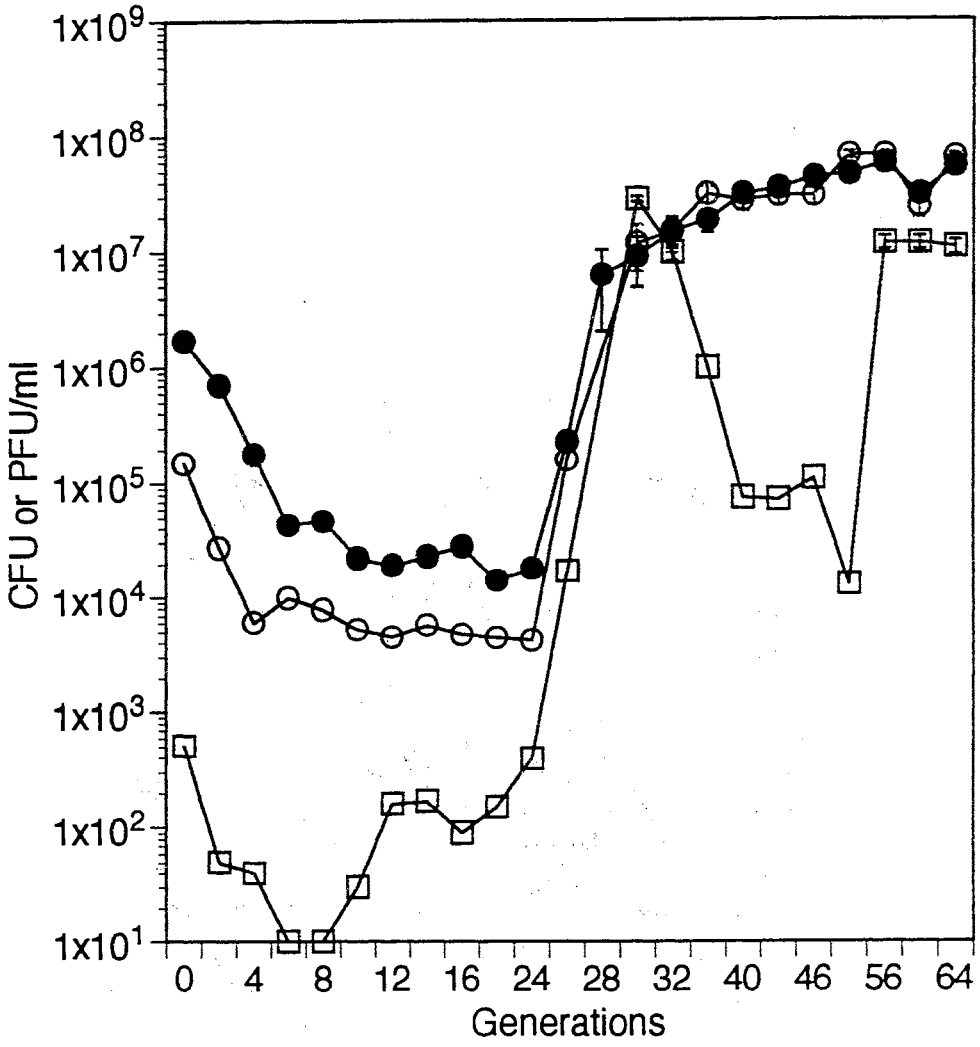


Fig. 17D. Chemostat microcosm incubated at a generation time of 12 hours. (O) RM132 (F116 lysogen), (●) PAO515, (□) Phage F116

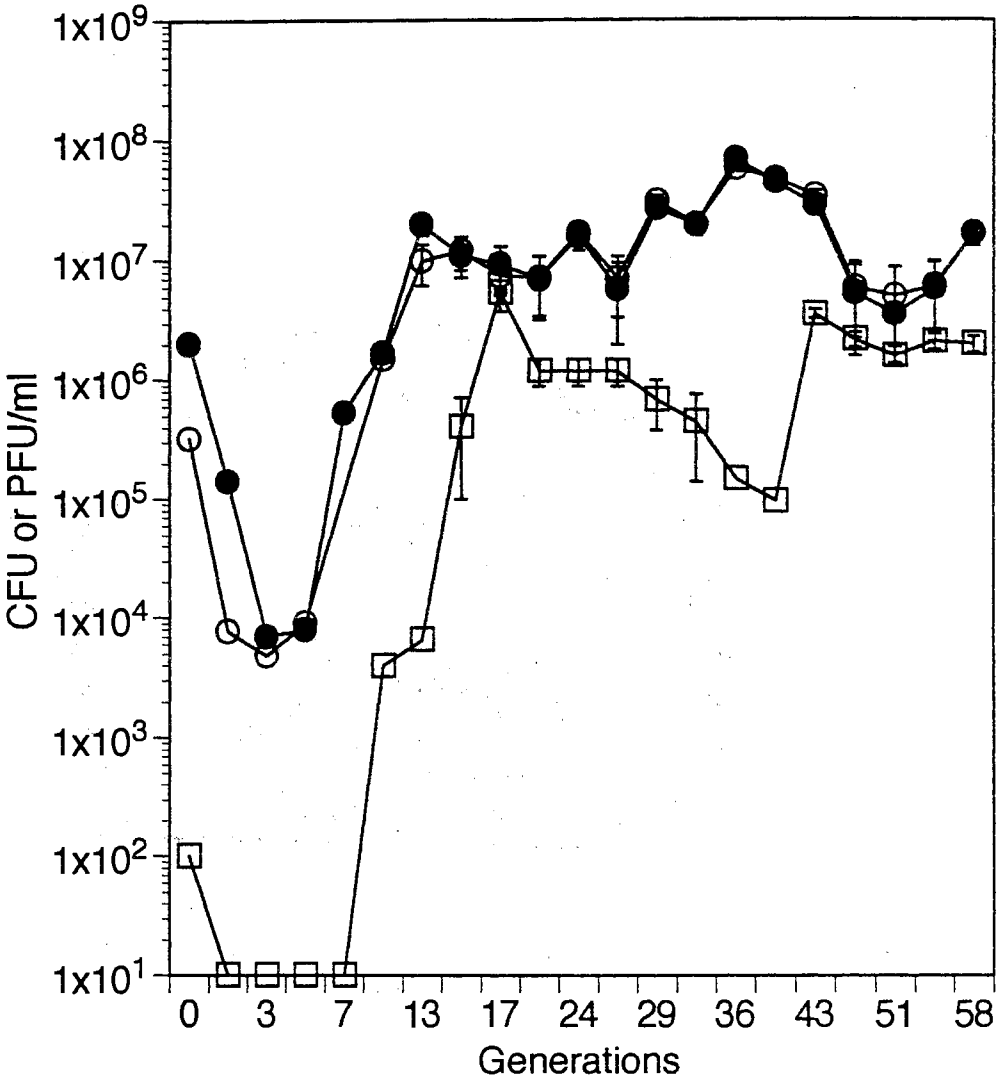


Fig. 17E. Chemostat microcosm incubated at a generation time of 14 hours. (○) RM132 (F116 lysogen), (●) PAO515, (□) Phage F116

At 5-and-8 hour generation times, phage F116 concentrations initially increased at a rapid rate, followed by a decline and another increase up to or surpassing host cell growth. Similar results were obtained at 10, 12, and 14 hour generation times except that phage concentrations initially decreased to well below inoculation levels. This was coincident with an initial drop in cell numbers, as well. At such long generation times, it would be expected that populations would require an initial period of acclimation before adequate growth could be established.

The oscillating phage concentrations seen in all of these cultures is a phenomenon typically associated with predator-prey growth in a closed, regulated system (16). Due to this oscillating nature, it is difficult to form a pattern when assessing PBRs. However, in general, phage production decreases as generation times extend (Table 15), and the number of pseudolysogens increase as generation times increase (Table 16). These results are virtually identical to those obtained for phage F116 at differing nutrient concentrations.

Estimation of bacteriophage UT1 activities at varying generation times. These chemostat runs differed from all others in that phages consistently grew at higher rates than cells (Fig. 18A-E), which, according to Levin *et al.* (38) is the mathematically correct response of a one resource, one prey, one predator chemostat system. Why the other chemostat runs did not behave according to the mathematical model is unknown. However, other studies have not confirmed this model either (13, 37), exemplifying the fact that

Days	Generation Time				
	5 hr	8 hr	10 hr	12 hr	14 hr
0	<1	<1	<1	<1	<1
1	3	2	<1	<1	<1
4	57	204	23	<1	<1
8	789	100	1357	3	<1
10	13	27	70	3	567
12	3	2	4	13	120
14	<1	<1	5	77	23
16	1	<1	2	526	2
19	12	10	<1	26	23
23	9455	2	268	4	2
26	4286	17	778	<1	77
27	3214	336	349	203	431
29	6324	410	ND	444	276
30	3521	833	127	164	244

Table 15. Phage-to-bacteria ratios (PBRs) ($x10^{-3}$) in chemostats inoculated with RM132 and PAO515 at varying generation times. ND, Not determined

Day	5 hr Generation			8 hr Generation			10 hr Generation			12 hr Generation			14 hr Generation		
	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated
0	1/220	100	0	2/210	100	0	1/280	100	0	1/320	100	0	1/150	100	0
9	48/300	96	4	41/290	100	0	148/250	93	7	2/140	50	50	6/170	17	83
19	60/350	98	2	46/380	98	2	245/390	92	8	210/490	80	20	403/1185	79	21
23	55/390	96	4	51/320	96	4	224/350	89	11	189/360	77	23	189/450	81	19
28	68/350	96	4	51/300	94	6	153/330	87	13	245/590	76	24	88/129	82	18

Table 16. Comparison of the percent of phage-releasing cells (% activated) to pseudolysogenic cells (% not activated) in chemostat microcosms inoculated with RM132 and PAO515 at varying generation times.

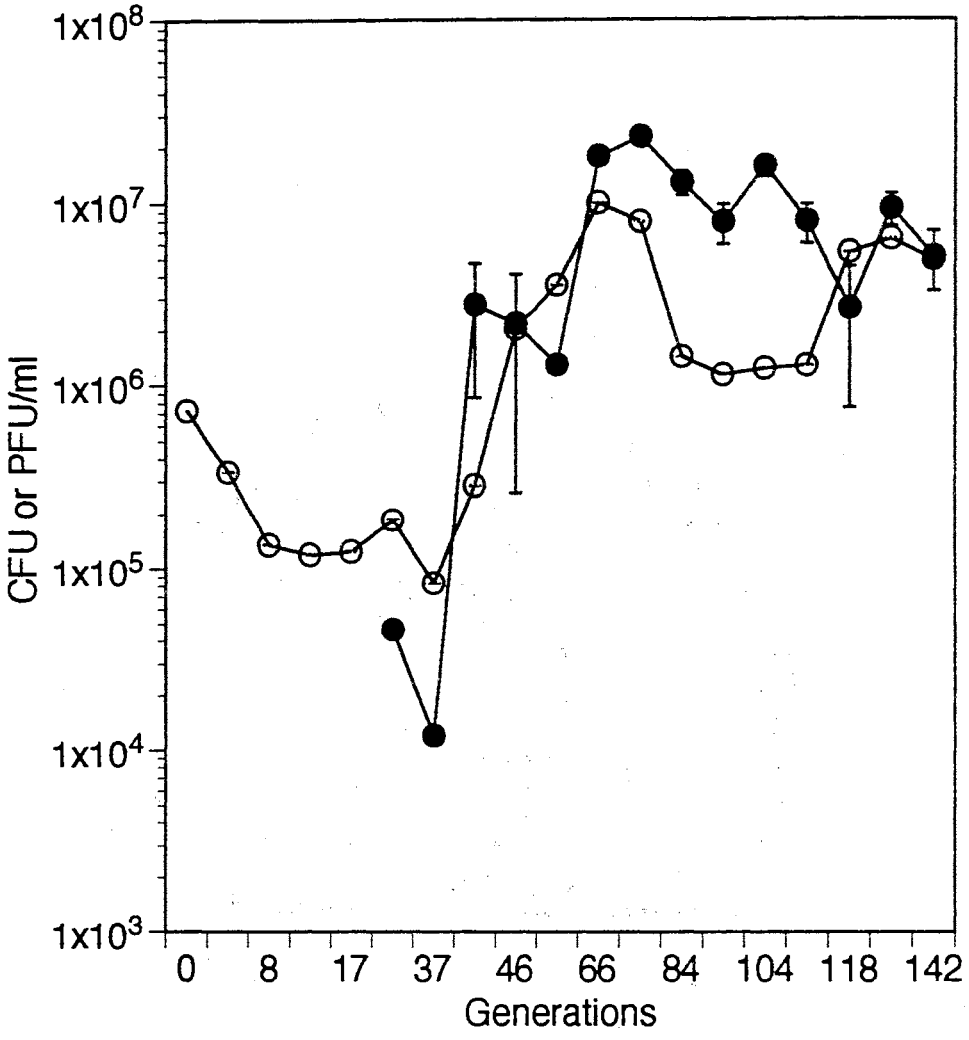


Fig. 18A. Chemostat microcosm incubated at a generation time of 5 hours. (O) PAO303, (●) Phage UT1

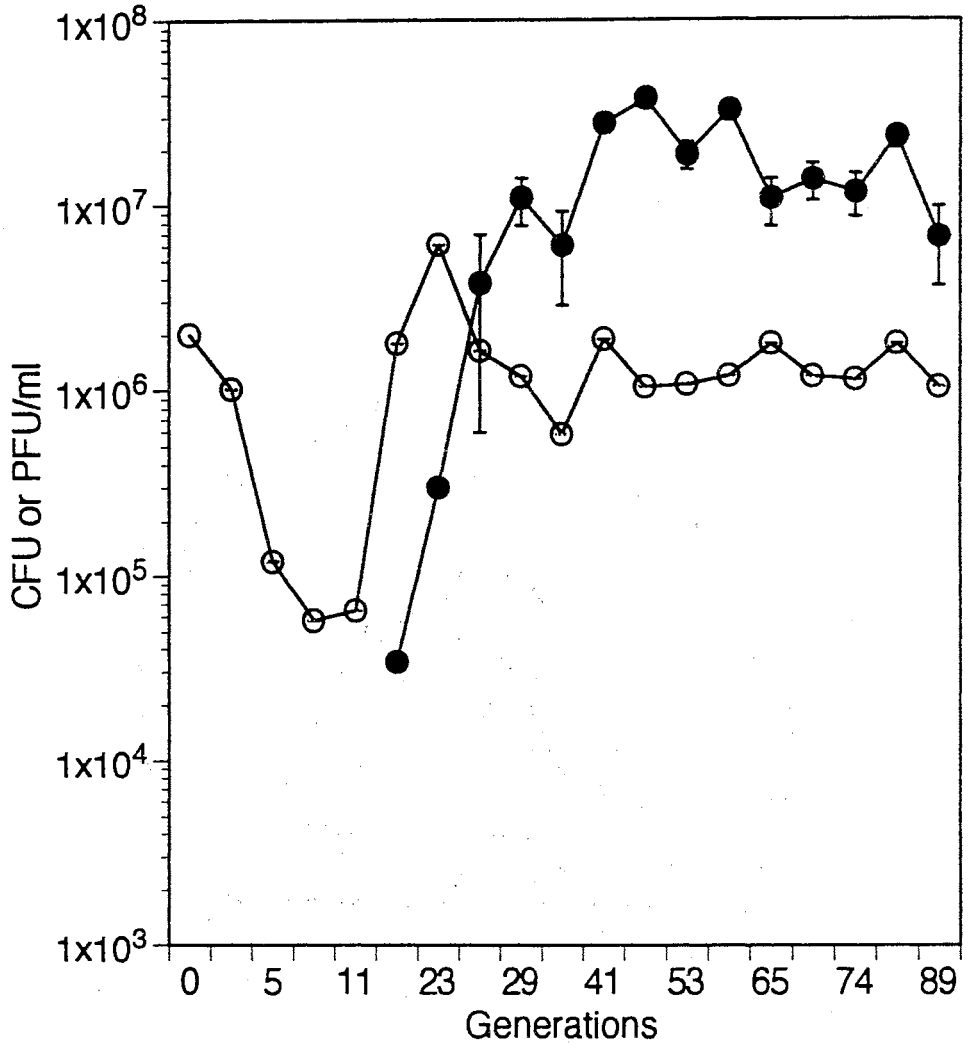


Fig. 18B. Chemostat microcosm incubated at a generation time of 8 hours. (○) PAO303, (●) Phage UT1

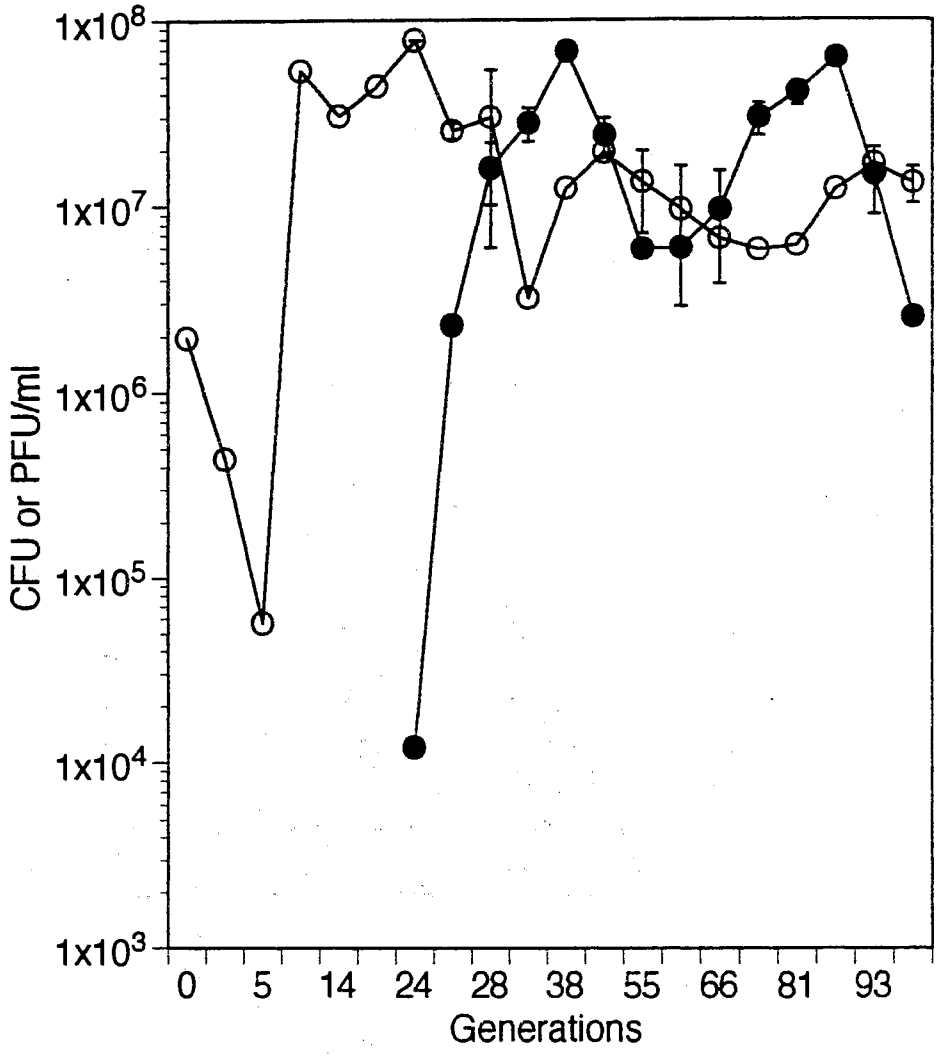


Fig. 18C. Chemostat microcosm incubated at a generation time of 10 hours. (○) PAO303, (●) Phage UT1

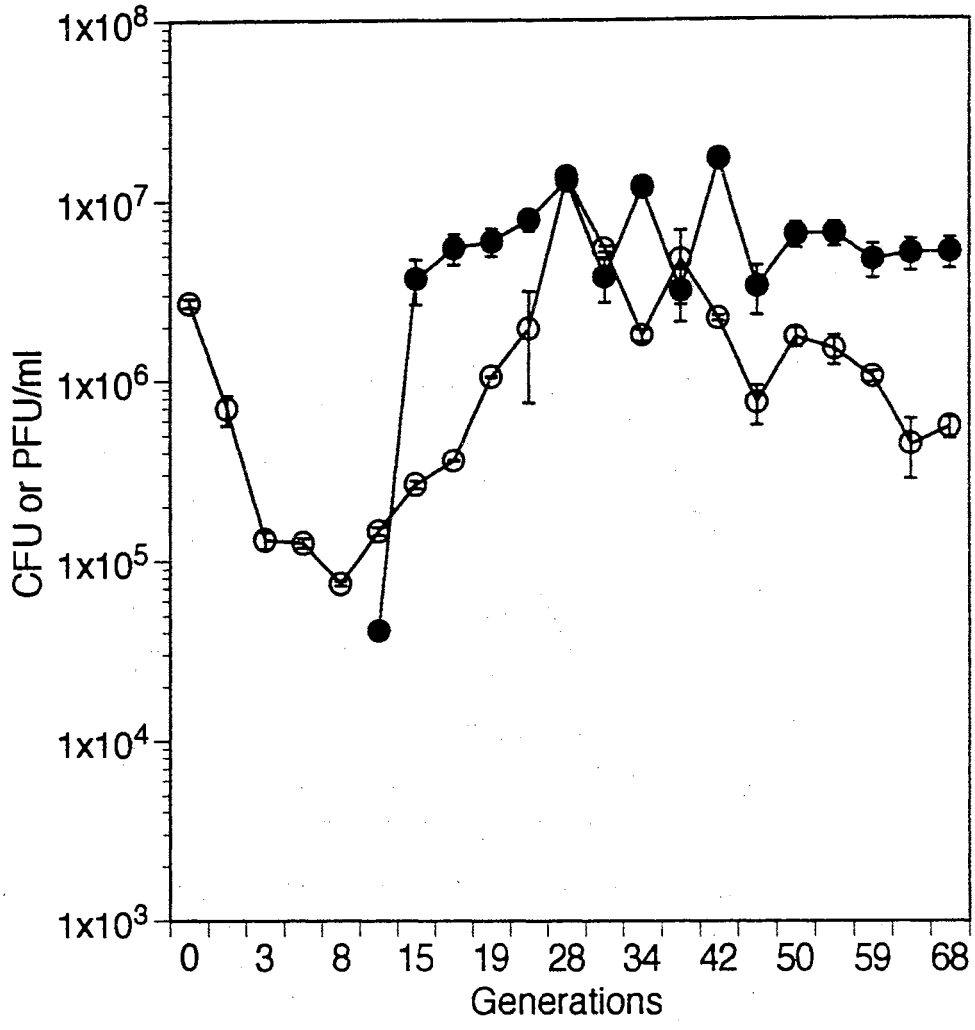


Fig. 18D. Chemostat microcosm incubated at a generation time of 12 hours. (O) PAO303, (●) Phage UT1

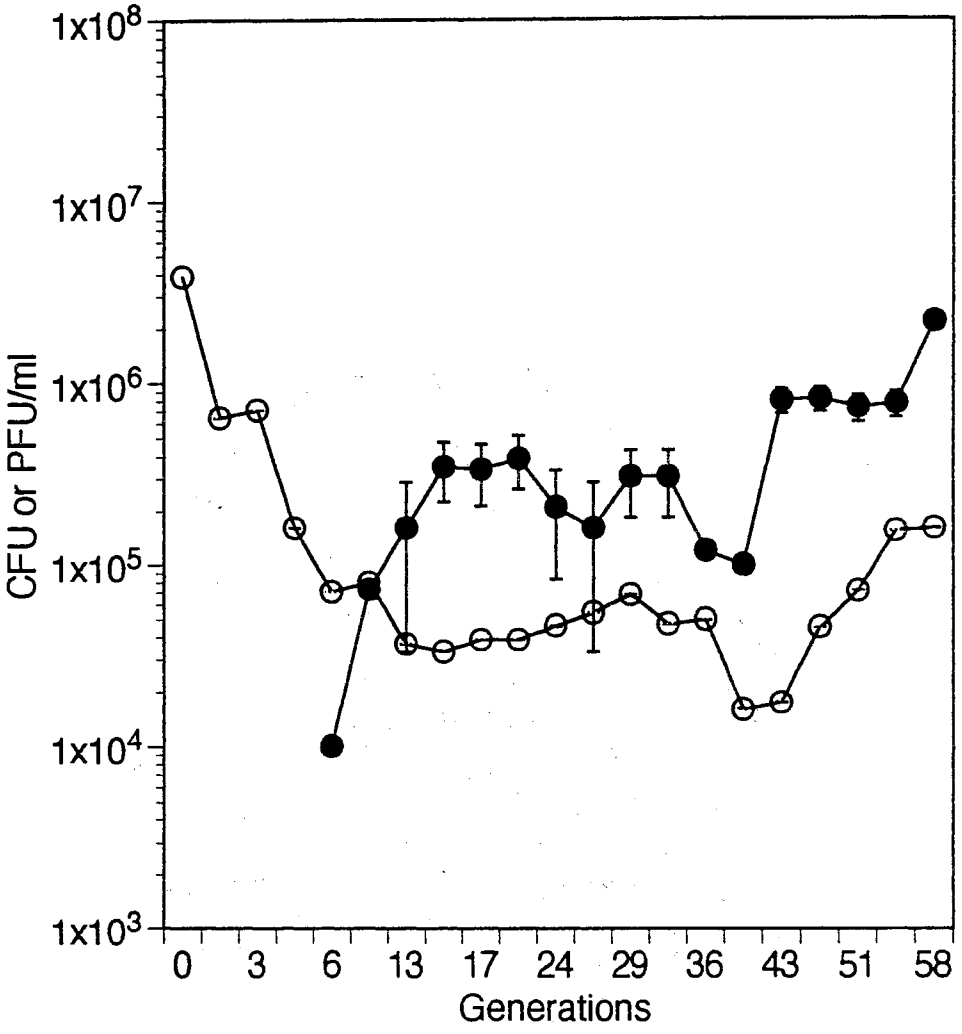


Fig. 18E. Chemostat microcosm incubated at a generation time of 14 hours. (○) PAO303, (●) Phage UT1

our understanding of predator/prey relationships under low nutrient conditions is severely lacking.

In any case, our concerns lie not in explaining these relationships, but with understanding pseudolysogeny. As such, it was again evident that pseudolysogeny occurred much more frequently at longer generation times when cells were more starved (Table 17). PBRs were fairly equal among all generation times (Table 18). But, once again, phage concentrations tended to oscillate, making an accurate analysis of the PBR difficult. Also, high PBRs at 12 and 14 hour generation times were caused by a decrease in cell numbers and not an overall increase in phage concentrations.

Conclusions

The temperate phage F116 behaved much more interestingly than the virulent UT1 phage. Phage F116 tended to follow classical Gause-type oscillations with a coincident reciprocal bacterial oscillation sometimes occurring as well (16). However, in Gause's experiments, oscillations routinely terminated in death of either the phage or its host. In these studies, phages tended to equalize with host populations, except under very low nutrient conditions when phage numbers maintained themselves at one-to-two-fold lower levels. This resulted in low PBRs. However, PBRs in general agreed with the PBRs generated in *in situ* lake water microcosms containing phage F116 (32, 76), assuring us that our data from chemostat experiments did correspond with *in situ* data.

Day	5 hr Generation			8 hr Generation			10 hr Generation			12 hr Generation			14 hr Generation		
	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated	Fraction	% activated	% not activated
9	482 /2800	89	11	32/160	80	20	0/330	0	0	9/150	57	43	2/120	50	50
16	270 /1100	84	16	307 /1200	76	24	162 /330	70	30	29/130	53	47	110/300	62	38
24	30/140	70	30	247 /1200	62	38	216 /1500	79	21	64/620	90	10	143 /1800	78	22
30	22/490	80	20	86 /1101	89	11	67/580	83	17	24/110	36	64	209/760	43	57

Table 17. Comparison of the percent of phage-releasing cells (% activated) to pseudolysogenic cells (% not activated) in chemostat microcosms inoculated with PAO303 and bacteriophage UT1 at varying generation times.

Days	Generation Time				
	5 hr	8 hr	10 hr	12 hr	14 hr
0	0	0	0	0	0
7	25	2	ND	28	91
8	14	5	ND	1396	438
9	982	230	ND	1528	1061
10	107	917	<1	571	883
13	178	1052	875	96	457
16	288	1474	544	70	294
17	897	3602	ND	667	453
20	696	2727	123	756	240
23	1280	611	44	450	625
29	145	1333	144	447	1824
30	105	660	ND	457	1028
31	ND	ND	513	1169	503
34	ND	ND	661	964	1375

Table 18. Phage-to-bacteria ratios (PBRs) ($x10^{-2}$) in chemostats inoculated with PAO303 and bacteriophage UT1 at varying generation times. ND, Not determined

The virulent UT1 phage consistently maintained its population in equilibrium with or above its host's. Only at a yeast extract concentration of 1 x 10^{-6}% did phage demonstrate a loss of equilibrium maintenance. PBRs remained more or less consistent between all experiments, except for transient epidemics where large phage increases were seen due to temporary decreases in host cell populations. Ogunseitan *et al.* (59), using phage UT1, found PBRs to range from <1 to 2742 in *in situ* experiments, which closely agrees with the PBRs generated here.

Assessment of pseudolysogen activity, as measured by the number of cells containing a phage genome but not actively releasing phage, was found to be highly dependent on nutrient concentration. As cells became more starved, pseudolysogen concentrations tended to increase. The effect of varying yeast extract concentrations 100-fold was more dramatic than varying generation times. Significant differences (Kolmogrov-Smirnov test, p=0.05 (77, 84)) were found between each group (10^{-2}%, 10^{-3}%, 10^{-4}%, 10^{-5}%, and 10^{-6}% yeast extract) in both the F116 and UT1 phage experiments. For generation times, no significant differences existed between the 5-and-8 hour generation times for both F116 and UT1. For UT1, no differences also existed between the 5 and 10, 8 and 10, and 12 and 14 hour generation times. Otherwise, all other groups significantly differed. The more pronounced effects generated by altering the nutrient concentration was more than likely due to a greater degree of variability between differing nutrient concentrations than what occurred for differing generation times. Pump speed was variable due to limitations of the chemostat itself, while nutrient addition was fairly exact.

Replicon *et al.* (67), using continuous culture chemostats, has recently developed a model identifying factors that influence transduction frequency under conditions typically encountered by microbial organisms in natural ecosystems. Transduction is a concern because of the potential for horizontal gene transmission from an introduced genetically engineered microorganism to members of the indigenous population. They concluded that transduction can stabilize a phenotype in a genetically heterogenous microbial population even if that phenotype occurs at a selective disadvantage. The rate of transduction was found to significantly correlate with the PBR. Our results further enhance this model by additionally showing that PBRs correlate with nutrient concentration and generation time. Therefore, as cells begin to enter a period of starvation and slow growth, PBRs will decrease as will transduction rates. However, due to the presence of pseudolysogenic cells, any influx of nutrient will dramatically elevate PBRs, thus increasing transduction rates as well. These effects are important when considering the application of GEMs to environmental systems. Stabilization of the introduced genetic material will result in an increased fitness of the released organism which will raise both its survivability potential and its probability for transferring this genetic material to the indigenous microbiota. Consequently, the potential risk of horizontal transfer of the engineered gene increases. These consequences must be taken into account before safe applications of GEMs can be conducted.

CHAPTER VII

CONCLUSION

This study began by first examining the process of transduction between a naturally isolated bacteriophage, UT1, and a naturally occurring freshwater host population. Previous studies by our laboratory have shown transduction to be a routine event in freshwater ecosystems (30, 49, 50, 67, 71, 76). The additional data presented here on phage UT1 clearly show that transduction between an indigenous phage and its indigenous hosts is prevalent, with frequencies of 10^{-4}-to-10^{-5} transductants/PFU being obtained. This is important information because it addresses our long-standing concern of horizontal gene transfer between natural microbial populations and GEMs. If we release GEMs into environmental ecosystems, we can no longer be assured that genetically altered material will not be transferred to the indigenous microbial population. In the early 1970's, General Electric genetically engineered a bacterial strain capable of oil degradation to be used for the bioremediation of oil slicks (39). It never performed as planned, generally being incapable of survival under typical environmental conditions long enough to adequately degrade the oil. However, it still managed to create an uproar with the general populace because of the common belief that this organism would eventually end up in

automobile gas tanks and Texas oil fields (23). Although far-fetched, concerns such as these must be acknowledged and all risks defined before the application of GEMs to environmental situations becomes a routine practice.

The second part of this study evolved from questions arising from the transduction experiments. Once it had been determined that transduction could occur in a freshwater environment with UT1, we began to wonder how a virulent phage like UT1 was able to survive in a natural system. Wouldn't a virulent phage eventually eradicate all its hosts and thereby eradicate itself? However, phage UT1 was found to exist in close equilibrium with its hosts, much as a temperate phage would. Therefore, a process similar to lysogeny must manifest itself with virulent phages as well. Consequently, this study addressed the previously ignored phenomenon of pseudolysogeny (3). Pseudolysogeny was found to occur with virulent phages, and temperate phages as well, under both laboratory and *in situ* conditions. The direct result of pseudolysogenic interactions is the extension of phage genomic half-lives, allowing bacteriophages to stably coexist with their host cells for longer durations than what was previously expected. This brings us back to transduction and the inherent risks of GEM applications. Due to pseudolysogeny, phage populations can remain in a dynamic equilibrium with their host populations, thus ensuring a greater probability of transduction events occurring and a greater risk of horizontal gene transfer.

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APPENDIXES

Isolation of plasmid DNA. Plasmid Rms149 was isolated from presumptive transductants by the rapid alkaline lysis method of Sambrook *et al.* (74). Isolates were grown overnight at 37°C in 5 ml LB containing carbenicillin at 700 ug/ml. The next day, 1.5 ml was transferred to a 1.5 ml microcentrifuge tube and pelleted at 16,000 x g for 1 min. The supernatant was discarded and the pellet resuspended in 100 ul of an ice-cold solution of 50 mM glucose, 10 mM disodium ethylenediaminetetraacetate (EDTA), 25 mM Tris-Cl (pH 8.0) and 4 mg/ml lysozyme. After standing for 5 min at room temperature, 200 ul of 0.2 N NaOH plus 1% sodium dodecyl sulfate (SDS) was added. The tube was gently inverted several times and incubated on ice for 5 min. One-hundred fifty microliters of an ice-cold potassium acetate solution (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H_2O) was then added and the tube vortexed and placed on ice for 5 min. The mixture was then centrifuged for 5 min at 16,000 x g and the supernatant transferred to a fresh tube. An equal volume of phenol/chloroform was added, vortexed, and centrifuged for 2 min. The supernatant fluid was once again transferred to a fresh tube to which was added two volumes of ethanol. After incubating 2 min at room temperature, the tube was centrifuged for 10 min. The supernatant was discarded and to the pellet was added 1 ml of 70% ethanol. After vortexing, the tube was once again centrifuged for 10 min. The supernatant was discarded and the pellet dried

briefly in a Savant Speed Vac. Fifty microliters of TE (10 mM Tris-Cl, 1 mM EDTA; pH 8.0) containing 20 μg RNase/ml was then added and the pellet resuspended. Samples were stored at -20°C.

Isolation of bacteriophage DNA. F116 and UT1 DNA were isolated by the method of Silhavy *et al.* (78). To 1 liter of LB medium was added 20 ml of a fresh overnight culture of PAO1 along with either bacteriophage F116 or UT1 at approximately 5 x 10^8 phage/ml. After shaking for 7 hours at 37°C, sodium chloride was added to 0.5 M along with 1 ml of chloroform and the entire culture centrifuged at 6,000 x g for 10 min. The supernatant fluid was removed and combined with polyethylene glycol (PEG) 6000 to 10% (w:v), placed on ice for 60 min, and centrifuged at 6,000 x g for 10 min at 4°C. The resulting phage /PEG pellet was resuspended in TM buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgSO_4$). The PEG was extracted by adding an equal volume of chloroform and centrifuging at 2,000 x g for 10 min. The upper aqueous layer containing the phage particles was transferred to a 13 ml Nalgene ultracentrifuge tube prepared with a glycerol step gradient (3 ml 40% glycerol overlaid with 3 ml 5% glycerol) and centrifuged at 100,000 x g for 60 min at 4°C in a 70 Ti rotor. The resulting pellet was resuspended in 1 ml TM buffer to which was added 10 μg RNase A/ml, 1 μg DNase I/ml, and 0.2 ml STEP buffer (0.5% SDS, 50 mM Tris·HCl pH 7.5, 0.4 M EDTA, 1 mg proteinase K/ml). After heating at 50°C for 15 min, the supernatant fluid underwent various extractions, first with Tris-saturated phenol, followed by phenol:chloroform:isoamyl alcohol (25:24:1),

and finally with chloroform:isoamyl alcohol (24:1). The final DNA concentration was determined photometrically at A_{260}.

Colony hybridizations. Colony hybridizations were performed with the Genius nonradioactive labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). All enzymes and buffers were purchased from Boehringer Mannheim as well. Probes were generated from F116 and UT1 DNA via random primed DNA labeling. Two microliters of heat denatured DNA (boiling, 10 min) was combined with 2 μl 10X hexanucleotide mixture (catalog #1277081), 2 μl dNTP labeling mixture (catalog #1277065), 14 μl dH_2O, and 1 μl Klenow enzyme (catalog #1008404). Reactions were incubated overnight at 37°C and terminated the next day by the addition of 2 μl 200 mM EDTA pH 8.0. The concentration of labeled DNA was estimated by comparison against standards supplied in the kit.

Samples to undergo hybridization were plated to near confluency on LB agar or on PMM plates containing arginine at 25 ug/ml and incubated approximately 15 hours at 37°C, after which they were held at 4°C for 1 hour. Magnagraph 85 mm diameter nylon membranes (Micron Separations, Inc., Westboro, MA) were then laid on the plates to initiate colony transfer. After 5 min, membranes were removed and placed colony side up on Whatman 3MM chromatography filters pre-wetted in denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 15 min followed by another 15 min incubation on a filter pre-wetted with neutralization solution (1.0 M Tris-HCl pH 8.0, 1.5 M NaCl). Membranes were then UV-crosslinked and placed into heat seal bags (Micro-Seal, Dazey

Corp., Industrial Airport, KS) with prehybridization solution (5X SSC (1X = 8.8 g NaCl, 4.4 g sodium citrate; pH 7.0), 1% Blocking reagent (catalog #1096176), 0.1% N-lauroylsarcosine, 0.02% SDS) and prehybridized at 65°C for 2 hours. New solution was added along with the denatured DNA probe (boil, 10 min) and incubation continued overnight at 65°C with slow shaking. The next day, membranes were washed twice in 2X wash solution (2X SSC, 0.1% SDS) for 5 min and twice in 0.5X wash solution (0.5X SSC, 0.1% SDS) for 15 min at room temperature.

Detection proceeded by blocking the membranes in Genius buffer 2 (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 2% Blocking reagent) for 1 hour. Anti-digoxigenin Fab fragments conjugated to alkaline phophatase (catalog #1093274) were then added followed by several washes. Reacting the bound antibody conjugate with Lumi-Phos 530 (catalog #1413155) resulted in production of the chemiluminescent signal which was detected within 30 min on Kodak (Eastman Kodak Co., New Haven, CT) XAR-5 X-ray film.

Acridine-orange direct count (AODC) assays. The AODC method has been described by Hobbie *et al.* (21). Nuclepore filters (25 mm, 0.2 μm pore size; Costar Scientific Corp., Cambridge, MA) were stained in Irgalan black solution (0.04 g Irgalan black (Ciba-Geigy Corp., Greensboro, NC), 0.4 ml acetic acid, 19.6 ml dH_2O) overnight to reduce background fluorescence and rinsed in distilled water several times before use. A 2 ml sample of cells was combined with 0.2 ml of 0.1% acridine orange (in dH_2O) and incubated for 2 min at room temperature. The solution was then poured into a 3 ml syringe and filtered

through the Nuclepore filter. The damp filter was placed on a microscope slide and a drop of immersion oil was sandwiched between the filter and a cover slip. Cells were examined with a Nikon Optiphot-2 fluorescent microscope under oil immersion at a magnification of 100X.

Cells grown at high growth rates will fluoresce red/orange due to the predominance of RNA over DNA (14). A larger number of acridine orange molecules intercalate into the looser RNA structure, allowing for the formation of acridine orange dimers which fluoresce red/orange. Inactive, starved bacteria, however, contain predominantly DNA and will fluoresce green. DNA has a more rigid structure, allowing fewer acridine orange molecules to intercalate, and, consequently, fewer dimers to be formed.

VITA

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Doctor of Philosophy

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